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THE PHYSIOLOGY *of* POLYAMINES

Volume II

Uriel Bachrach
Yair M. Heimer

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The Physiology of Polyamines

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Volume II

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PREFACE

More than 300 years ago Antoni van Leeuwenhoek described crystals of biological materials derived from human semen. A further 250 years were to pass before the structure of these substances, which are now known as spermine and spermidine, has been established. During the 20th century, these amines, as well as other polyamines, have been isolated from practically every microbial, plant, or animal cell analyzed. Subsequently, their metabolic pathways and the various enzymes involved in polyamine synthesis and degradation have been characterized.

Recently, the importance of polyamines has been recognized because of their close relationship to cell proliferation and carcinogenesis. This led to an explosive growth in publications on polyamines, on their biosynthetic enzymes, and data on their physiological functions. During the past decade more than ten International Polyamine Meetings took place. The proceedings of those meetings were published in various books, including the four-volume *Advances in Polyamine Research* (Raven Press, New York).

The diversity of the aspects of polyamine research makes it practically impossible for a single author to summarize the present state of art in the field. In these volumes, the various aspects are reviewed by experts, including molecular biologists, biochemists, botanists, and clinicians.

We believe that they will provide useful information to those who are interested in the study of growth regulation and differentiation in normal and neoplastic systems and will stimulate further research in the field, mainly by attracting the attention of students and young scientists.

It is our hope that this book will also help in clarifying some mysteries of nature and contribute to the welfare of mankind.

The Editors
March 1988

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SERIES TABLE OF CONTENTS

Volume I

Polyamines and Steroid Sex Hormone Action
Polyamines and Human Reproduction
Polyamines and Heart Physiology
Polyamines, Hormone Receptors, and Calcium Fluxes
Polyamines and Cell Differentiation
Polyamines in Tissue Regeneration
Specificity and Binding in Polyamine/Nucleic Acid Interactions
Polyamines and Initiation of Protein Synthesis in Eukaryotes
Polyamines and RNA Synthesis
Effects of Polyamines and Antibiotics on the Structure and Function of Ribosomes
Acetylation and Interconversion of the Polyamines
Oxidation of Diamines and Polyamines
Polyamine Oxidases and Oxidized Polyamines
5′-Deoxy-5′–Methylthioadenosine: Novel Metabolic and Physiological Aspects
The Physiological Significance of Ornithine Decarboxylase
Molecular Genetics of Mammalian Ornithine Decarboxylase
The Molecular Biology of Mouse Ornithine Decarboxylase
Inhibitors of Ornithine and *S*-Adenosylmethionine Decarboxylases
The Roles of Antizyme in the Regulation of Ornithine Decarboxylase Activity in EukaryoticCells
S-Adenosylmethionine Decarboxylase: Genes and Expression

Volume II

Polyamines and the Growth of Bacteria and Viruses
Polyamines in Thermophiles
Propylamine Transfer Reactions in Thermophilic Archaebacteria: Enzymological Aspects and Comparative Biochemistry
Microbial Mutants Deficient in Polyamine Synthesis
Regulation of the Putrescine Biosynthetic Genes (*speA*, *speB*, and *speC*) in *Escherichia coli*
Utilization of L-Arginine, L-Ornithine, Agmatine, and Putrescine as the Major Source of Nitrogen and Its Control in *Escherichia coli* K-12
Polyamines and Plant Response to Stress
Polyamines and Plant Growth and Development
Polyamines and Plant Hormones
Polyamines in Plant Mutants
Polyamines in Health and Disease
Polyamine Metabolism and Neoplastic Growth: A Programmed Deregulation?
Polyamines as Markers of Malignancy
Polyamines as Indicators of Disease Activity and Response to Therapy in Cancer Patients
Inhibitors of Polyamine Biosynthesis as Therapeutic Agents
Clinical Studies of α-Difluoromethylornithine and α-Interferon Combination in Cancer Patients
Changes in Polyamine Metabolism in Tumor-Bearing Hosts with Total Parenteral Nutrition and Intravenous Infusion of α-Difluoromethylornithine

TABLE OF CONTENTS

SECTION A. POLYAMINES IN MICROBES

Chapter 1
Polyamines and the Growth of Bacteria and Viruses 3
A. Stanley Tyms

Chapter 2
Polyamines in Thermophiles 35
Tairo Oshima

Chapter 3
Propylamine Transfer Reactions in Thermophilic Archaebacteria: Enzymological Aspects and Comparative Biochemistry 47
Giovanna Cacciapuoti, Marina Porcelli, Gabriele Pontoni, and Vincenzo Zappia

Chapter 4
Microbial Mutants Deficient in Polyamine Synthesis 63
Celia White Tabor and Herbert Tabor

Chapter 5
Regulation of the Putrescine Biosynthetic Genes (*speA*, *speB*, and *speC*) in *Escherichia coli* 73
Stephen M. Boyle, Jonathan M. Wright, C. Satishchandran, and Jagdeep Buch

Chapter 6
Utilization of L-Arginine, L-Ornithine, Agmatine, and Putrescine as the Major Source of Nitrogen and Its Control in *Escherichia coli* K-12 85
Yeheskel S. Halpern and Esther Metzer

SECTION B. POLYAMINES AND PLANTS

Chapter 7
Polyamines and Plant Response to Stress 99
Arthur W. Galston

Chapter 8
Polyamines and Plant Growth and Development 107
Nello Bagni

Chapter 9
Polyamines and Plant Hormones 121
Arie Altman

Chapter 10
Polyamines in Plant Mutants 147
Russell L. Malmberg and Andrew C. Hiatt

SECTION C. POLYAMINES AND DISEASES

Chapter 11
Polyamines in Health and Disease 163
Robert A. Campbell, Zoe Hunt-Retzlaff, and J. Beth Russi

Chapter 12
Polyamine Metabolism and Neoplastic Growth: A Programmed Deregulation? 183
Giuseppe Scalabrino and Maria E. Ferioli

Chapter 13
Polyamines as Markers of Malignancy 219
Takatoshi Matsumoto and Osamu Suzuki

Chapter 14
Polyamines as Indicators of Disease Activity and Response to Therapy in Cancer Patients 235
Uriel Bachrach

Chapter 15
Inhibitors of Polyamine Biosynthesis as Therapeutic Agents 251
Juhani Jänne and Leena Alhonen-Hongisto

Chapter 16
Clinical Studies of α-Difluoromethylornithine and α-Interferon Combination in Cancer Patients 287
M. Talpaz, K. Nishioka, and J. Gutterman

Chapter 17
Changes in Polyamine Metabolism in Tumor-Bearing Hosts with Total Parenteral Nutrition and Intravenous Infusion of α-Difluoromethylornithine 293
Kenji Nishioka, V. Bruce Grossie, Jr., David M. Ota, and Jaffer A. Ajani

INDEX 303

Section A.
Polyamines in Microbes

Chapter 1

POLYAMINES AND THE GROWTH OF BACTERIA AND VIRUSES

A. Stanley Tyms

TABLE OF CONTENTS

I. Introduction 4

II. Polyamines and Bacteria 4

III. Polyamines and Viruses 6
  A. Polyamine Biosynthesis in Animal Cells 6
  B. DNA-Containing Viruses 7
  C. RNA-Containing Viruses 10

IV. Polyamines and *Mycoplasma* 12

V. Physiological and Biochemical Function of Polyamines 14
  A. Bacterial Systems 16
  B. Virus-Infected Cells 17

VI. Clinical Application of Polyamine Biosynthesis in Microbiology 20
  A. Polyamine Inhibitors as Antimicrobial Agents 20
  B. Antiviral Activity 21
  C. Other Considerations 22
  D. Antibacterial Agents 23
  E. Polyamines as Clinical Markers for Microbial Infections 24

VII. Concluding Remarks 25

Acknowledgments 26

References 26

I. INTRODUCTION

The term bacteria is generally applied to a heterogeneous group of unicellular organisms that are minute and relatively simple in organization. There is no intention to enter into details on the classification of prokaryotes except to say that they are primitive organisms that lack an organized nucleus and fall between the lower plants (algae and fungi) and lower animals (protozoa). The association of polyamines with these relatives of bacteria are dealt with elsewhere in this book and will not be discussed in this chapter.

There is no doubt that the free-living bacteria make a major contribution to the existence of higher animals in terms of biology and medicine with equally important applications in industry. Contrary to common belief, very few bacteria are capable of causing disease in man or animals. In relation to polyamines, the clinical aspects have importance but bacteria are perhaps most useful as genetic tools for the study of the metabolic pathways.[1]

The early work on polyamines and microorganisms involved the study of putrefied tissues. In 1910, Achermann described the use of decomposed tissue to convert the amino acids ornithine to putrescine and lysine to cadaverine, although eventually it was shown that putrescine could be derived from arginine. The biodegradation of amino acids appeared to be a protective mechanism in the control of intracellular acidity rather than an essential need for the decarboxylation. Subsequently, it turned out that the products of decarboxylation of amino acids and their derivatives were growth factors for certain bacteria.

It was established some time ago that bacterial viruses (T-even bacteriophage) contained polyamines in amounts sufficient to neutralize about 50% of the negatively charged phosphate groups of the viral DNA.[2,3] Viruses in the free state are, in essence, inert particles which have the means to reproduce themselves but only on reaching an intracellular environment. As is well known, such infections are a major cause of morbidity and mortality in man and animals. With relatively little genetic information, viruses (either DNA- or RNA-containing) can infect and destroy cells, induce persistent or latent infections, and many have the ability to integrate the genome within host-cell chromosomes resulting in immortalization of cells. Due to the limited genetic information, viruses are obligate intracellular parasites with an almost total dependence on cellular metabolic functions. This intimate association between the virus and the host cell makes chemotherapy much more difficult to achieve when compared with other free-living microbes. On the other hand, viruses have long been important in the study of the expression of foreign genes in animal cells; most recently this has been applied to the study of polyamines.[4,5]

The major theme of these discussions has been directed toward polyamines and microbial infections in the context of potential clinical application. The two areas of major interest relate to inhibitors of the pathway as antimicrobials and the presence of polyamines or their derivatives as markers of disease. Equally important may be the role of these molecules in microbial pathogenicity by modulation in the immune response.[6] Our major research interest is in the study of human herpesviruses, in particular the cytomegaloviruses (CMVs). Unlike many other viruses of importance to man in terms of disease, CMV up regulates the host cell after infection.[7] This appears to be obligatory for virus growth and reflects on the importance of polyamine metabolism. Due to the prominence of CMV as a cause of disease and our personal knowledge of polyamines in the growth of this virus, a good deal of discussion is centered on this topic.

II. POLYAMINES AND BACTERIA

Both putrescine and spermidine are essential factors in the growth of *Haemophilus influenzae*,[8] *Neisseria perflava*,[9] *Pasteurella tularensis*,[10] *Lactobacillus casei*,[11] and *Veillonella alcalescens*.[12] Putrescine, spermidine, and other amines have been associated with a wide variety of microorganisms but the presence in bacteria of spermine, the aminopropyl derivative of spermidine, was only rarely documented.[13] More recently, the tetraamine and other unusual polyamines have been identified in numerous bacteria. These include norspermidine, homospermidine, norspermine, thermospermine, caldopentamine, and homocaldopentamine.[14]

Prokaryotes have at least two alternative pathways for the synthesis of putrescine involving the decarboxylation of ornithine and arginine. Work by Morris and Pardee[15] showed that, unlike eukaryotes, *Escherichia coli* in fact expressed two decarboxylases specific for ornithine. A degradative enzyme, first described by Gale[16] is involved in the control of pH and a biosynthetic enzyme is the major enzyme activity in the formation of putrescine. Although both enzyme activities, such as amino acid decarboxylases in general, are pyridoxal phosphate requiring, they were distinguishable in *E. coli* by other cofactors and by immunological reactivity.

A second major pathway for the synthesis of putrescine by *E. coli* involves the decarboxylation of arginine, which is produced from ornithine by the action of arginase in the urea cycle. The product of this reaction is agmatine, which is converted to putrescine directly by the enzyme agmatinase or through the action of agmatine iminohydrolase and putrescine transcarbamylase.[17] The arginine decarboxylase (ADC) activity can also be separated into degradative and biosynthetic. They can be distinguished by different optimal pH for reactions but also by a differential requirement for magnesium ions in the case of the biosynthetic enzyme. The recent availability of highly specific inhibitors of ornithine decarboxylase (ODC) and ADC has enabled the study of differential inhibition of these enzymes in *E. coli* and *Pseudomonas aeruginosa*.[18] In conditions of almost 100% inhibition of ODC, putrescine levels were maintained by the alternative agmatine route. Interestingly, inhibition of both carboxylase activities, thus reducing putrescine levels, was offset by an effect of increased spermidine availability. The interrelationship of the two decarboxylases is probably a highly regulated phenomenon, almost certainly directly influenced by arginine metabolism.

A third important amino acid, L-methionine, is a key molecule in the extension of the diamino butane molecule to produce spermidine. *S*-Adenosyl methionine is converted to *S*-adenosyl-5′-deoxy-(5′)-3-methylthiopropylamine by the action of *S*-adenosylmethionine decarboxylase (SAMDC). Unlike other decarboxylases, this enzyme does not require pyridoxal phosphate as a cofactor but the native enzyme has one molecule of covalently bound pyruvate.[19] Some bacteria utilize L-aspartic β-semialdehyde instead of decarboxylated *S*-adenosylmethionine as the aminopropyl donor.[14] The formation of spermidine is completed by the transfer of the aminopropyl moiety to putrescine. The amino transferase (spermidine synthase) from *E. coli* comprises two identical subunits[20] and requires intact sulfhydryl groups for activity. This amino transferase is highly specific for putrescine at physiological pH and appeared unable to synthesize spermine by the same reaction with spermidine as the recipient molecule.

Aminopropyl transferase activity with affinity for both putrescine and spermidine has been identified in a number of *Acetobacteria*.[21] These organisms contain both spermidine and spermine, but it is not known if one or two transferases are involved (see Section III.A.). *Thermus thermophilus* produces at least 11 polyamines of which 9 are rare or unusual.[22] The tetraamine thermospermine was present but not its isomer spermine,[23,24] although both tetraamines have been identified recently in *Agrobacterium tumefaciens*.[14] In some bacteria, polyamines are present that consist only of propyl groups, namely, 1,3 diaminopropane, norspermidine, norspermine, and caldopentamine[25,26] (for details see Chapters 2 and 3).

In *E. coli*, the other product generated in stoichiometric amounts during the synthesis of spermidine is 5′-methylthioadenosine (MTA). This molecule is known to influence the aminotransferase activity by allosteric means but is readily hydrolyzed for recovery of the nucleoside and 5′-methylthioribose.[27]

III. POLYAMINES AND VIRUSES

The DNA-containing viruses found in man, herpes simplex virus (HSV), CMV, and vaccinia virus have been shown to contain significant amounts of spermidine and spermine (see below). The equalization of certain charged groups associated with nucleic acid by the positively charged polyamines to allow conformational changes is one obvious function for these substances in the replication of viruses, and this is discussed later. Animal viruses with RNA as the viral genome such as myxoviruses[28] and picornaviruses[29,30] are also reported to contain putrescine, spermidine, and spermine. It is quite likely that polyamines have a greater or lesser association with most animal viruses, although the relationship of their biosynthesis to virus growth after infection has only been studied in a few cases. Details of this work are considered below but first something needs to be said about polyamine biosynthesis in uninfected cells.

A. Polyamine Biosynthesis in Animal Cells

Two major differences exist between polyamine biosynthesis in eukaryotes and prokaryotes. The decarboxylation of ornithine is the only known anabolic route for the synthesis of putrescine and a second aminopropyl transferase, spermine synthase, is present which is specific for the formation of spermine. Interestingly, spermine is the major polyamine component of the nucleus in animal cells.[31] This may be significant in evolutionary terms since, as discussed earlier, an aminopropyl transferase activity in some prokaryotes can generate spermine (or other tetraamines) and this may have led to the generation of isoenzymes in primitive organisms. Characterization of each enzyme from bovine brain and rat prostate suggests a dimeric composition of 70,000 to 80,000 daltons in molecular weight.[32] The two distinct polyamine synthases exhibit no requirement for cofactors or prosthetic groups. Spermine synthase, and to a much lesser extent spermidine synthase, is subject to marked inhibition by MTA, the other product of the synthase reactions.[33] It seems unlikely that MTA is utilized through a series of reverse reactions to generate spermidine and putrescine from spermine. This is an important consideration if the pathway is to be exploited for the chemotherapy of proliferative disease.

It is now clear that a catabolic pathway is available which uses oxidizing activities,[34] giving rise to the notion that polyamine metabolism is a cyclic process.[35]

Interestingly, the level of MTA in mammalian cells is extremely low compared to *S*-adenosylmethionine and polyamines. Estimates per gram of rat tissue by reverse-phase HPLC[36] or radioimmune assay[37] were in nanomole quantities and this included the ventral prostate, an organ known to contain polyamines at levels three orders of magnitude higher.[38,39] An MTA phosphorylase is responsible in a variety of eukaryotes for the degradation of MTA to 5′-methylthioribose-1-phosphate and adenine,[27] and it seems unlikely that MTA has a major regulatory function in the synthesis of polyamines.

As is the case in bacteria, ODC is a key regulatory enzyme in this pathway with a very short half-life (minutes). This enzyme is inhibited competitively by its product putrescine and to a much lesser extent by both polyamines. Control by a novel regulatory protein appears to have major influence on the function of this enzyme. The ODC-antizyme(s) complex has been found in a range of mammalian cells and tissues[40-42] as well as in *E. coli*.[43,44] The two antizymes, designated antizyme 1 and antizyme 2, characterized for *E. coli* inhibit only the biosynthetic ODC (and ADC) and not the biodegradative enzymes. This suggests major regulatory properties in *E. coli*.[44]

Of most interest were the findings that these antizymes have inhibitory properties against rat liver ODC and have amino sequences in common with two basic ribosomal proteins.[45] Although the interaction of antizyme protein(s) with ODC appears to be important in regulating this key enzyme, the subtle response to putrescine levels[46] and the possibility of ionically distinct forms of ODC may also have important roles.[47]

Spermidine is synthesized from putrescine in much the same way as the reactions described for prokaryotes. SAMDC, unlike the corresponding enzyme from *E. coli*, is activated by putrescine and lacks the requirement for magnesium ions. Both enzyme activities have pyruvate as a prosthetic group. This enzyme must be considered of equal importance to ODC in the regulation of the pathway, in part due to its short half-life (80 min). An initial phase of enzyme activity was associated with *de novo* protein synthesis but the second phase corresponded to an extension of the half-life of the enzyme activity.[48] Regulation of this decarboxylase may also occur through its product.[49]

The polyamine synthases activities have long half-lives[50] and appear to have little influence on the rates of metabolism; therefore the decarboxylases provide prime targets for chemotherapy. Although neither aminopropyl transferase reaction is rate limiting, specific inhibitors of these enzymes may be important in regimens involving drug combinations.

B. DNA-Containing Viruses

A feature of many animal viruses during the various strategies for virus replication is the ability to down regulate the synthesis of the major cellular macromolecules. Poxviruses are the largest of the animal viruses, approximately 220 × 350 nm in size and certain structural proteins of the virion have associated enzyme activities. These enzymes along with those expressed early in virus infection enable the virus to dispense with many of the host enzymes and, in fact, poxviruses rapidly cause a cessation in host-macromolecular synthesis. In 1975, Hodgson and Williamson[51] described the induction of ODC activity in cells infected with vaccinia virus. This was obviously surprising in view of the rapid shutdown in host-protein synthesis and the remarkably short half-life of all known ODC activities. The Km value for the virus-associated enzyme (0.05×10^{-3} m*M*) was significantly different from enzyme present in uninfected cells (0.12×10^{-3} m*M*) and this and other characteristics strongly suggested a novel, virus-coded enzyme. It was subsequently shown that the expression of ODC and activation of polyamine biosynthesis was indeed obligatory for the replication of vaccinia virus.[52,53] It was already known that vaccinia virus-specific enzymes were involved in arginine metabolism[54,55] and subsequently it was established that arginine was the source of ornithine for the synthesis of spermidine and spermine in virus-infected cells.[56]

As stated earlier, HSVs, like poxviruses, are known to contain spermidine and spermine within the virus particle. Also, like poxviruses, herpes simplex infection causes a major shutdown in host protein, RNA and DNA synthesis. Unlike vaccinia virus, herpes simplex virus inhibits rather than induces ODC activity[57,58] and consequently the synthesis of spermidine and spermine.[59,60] Herpes viruses can be readily fractionated into two distinct parts; an outer membrane-like structure (viral envelope) along with the tegument components are readily separable from the nucleocapsid core. The latter consists of a protein coat protecting the viral genome, in this case a molecule of double-stranded DNA. Spermine was associated with the nucleocapsid components (assembled in the nucleus) and spermidine with the envelope components (generated at the nuclear membrane/cytoplasm). The available evidence points toward the fact that polyamines extant within the cells suffice for the replication of herpes simplex viruses. Only by depletion of polyamines prior to infection can virus replication be modulated.[53,59-62]

In the absence of anabolic synthesis of putrescine, spermidine, and spermine during HSV growth, it is possible that the di- and triamines are generated by catabolic means. It was recently established that polyamine oxidase, which is found in liver peroxisomes, oxidizes spermine to spermidine and subsequently spermidine to putrescine. In the process, both diaminopropane moieties are converted to 3-aminopropionaldehyde and this proceeds more efficiently if the polyamines are first acetylated: this is achieved by spermine or spermidine N′-acetyl transferase.[63] As reviewed by Williamson and Tyms,[4] the consistent finding that spermidine increases relative to spermine after in vitro infections with HSV is difficult to explain in the absence of anabolic biosynthesis of spermidine. For example, in baby hamster kidney (BHK 21) cells infected with HSV, the spermidine:spermine ratio was shown to increase from 0.66 in the early phase of virus growth to greater than one at late times.[64] Both acetyl transferase and polyamine oxidase activities have been identified in BHK 21 cells and may constitute an important route for the generation of spermidine and putrescine during cell growth.[65] Specific inhibitors of polyamine oxidase are now becoming available and could determine important mechanisms for antiviral activity.

HSV can cause serious and life-threatening illness but more often this virus is known for its ability to establish latent infections. Recrudescence occurs at particular dermatomes after reactivation of virus in nerve root ganglia.[66] Another herpes virus, human CMV, also persists in the body after primary infection. This results in a range of conditions from asymptomatic infections to severe complications including serious neurological damage. Human CMV is of major interest in terms of the physiology of polyamines due to an ability to energize rather than inhibit the host cell after infection. CMV infection induces cellular DNA,[67] RNA, and protein synthesis.[7] One consequence of the activation of host-macromolecular synthesis is the induction of ODC[58] and a marked increase in spermidine and spermine biosynthesis.[53,60] The increase in total spermine content of human embryo fibroblast cells when compared to uninfected cells was recorded from 24 hr post infection and high levels of the polyamine correlated with the growth of infective virus (Figure 1). It can be seen that spermidine levels in the infected cells also increased over the controls but a distinct characteristic of CMV infection was the reduction in the ratio of spermidine to spermine. These data were acquired using the prototype strain AD169 which has been extensively passaged in cell culture. Interpretation of results acquired with laboratory strains of CMV can be complicated by simultaneous infection with *Mycoplasma* species harbored within virus stocks (Section IV). Results in Table 1 show the induction of the polyamine pathway to be a consistent feature associated with the replication of five low-passage isolates of human CMV. The viruses were classified as human CMV and epidemiologically unrelated by restriction enzyme analysis of the viral genome.[68] The Colburn strain of CMV has been identified as simian-like, although originally isolated from the brain of a child.[69] This virus is readily distinguished from strains of human CMV by genome analysis (Figure 2) and by the relative molecular weight of virus-coded proteins. Infection of human embryo fibroblast (HEF) cells with the Colburn strain also resulted in an induction in polyamine biosynthesis[70] although the sensitivity of this virus to inhibition by α-difluoromethylornithine (DFMO), the irreversible inhibitor of ODC, was marginal when compared with human CMV.[62] All viruses classified as CMVs so far examined, which include murine CMV,[53] have the capacity to induce polyamine biosynthesis after infection.

The temporal response of ODC induction after HEF cells were infected with human CMV was completely different from the periodic nature of the enzyme observed after stimulation of uninfected cells with serum-containing medium.[58] By 24 hr, enzyme activity had declined to 10% of the peak value in serum-treated cells while in the CMV-infected cells activity remained high. This apparent alteration in the regulation of the virus-induced enzyme was confirmed by a lack of inhibition when infected cells were fed putrescine. Similarly, ODC expressed in CMV-transformed cells was less sensitive to inhibition by polyamines than normal cells.[72] The response was different for putrescine and spermidine, and varied with the extent of growth of the transformed cells. We have now demonstrated that the activity of the polyamine pathway is continuous in CMV-infected cells as long as cells remain highly productive for infective, progeny virus.[71] Most often this was for periods in excess of 10 days postinfection.

Aberrant regulation of cellular functions after virus infection is not unusual. Adenoviruses, like human CMV, are a group of DNA-containing viruses which, upon infection, cause a progression of G1-arrested cells into the S phase.[73,74] Both adenovirus[75] and human CMV[76]

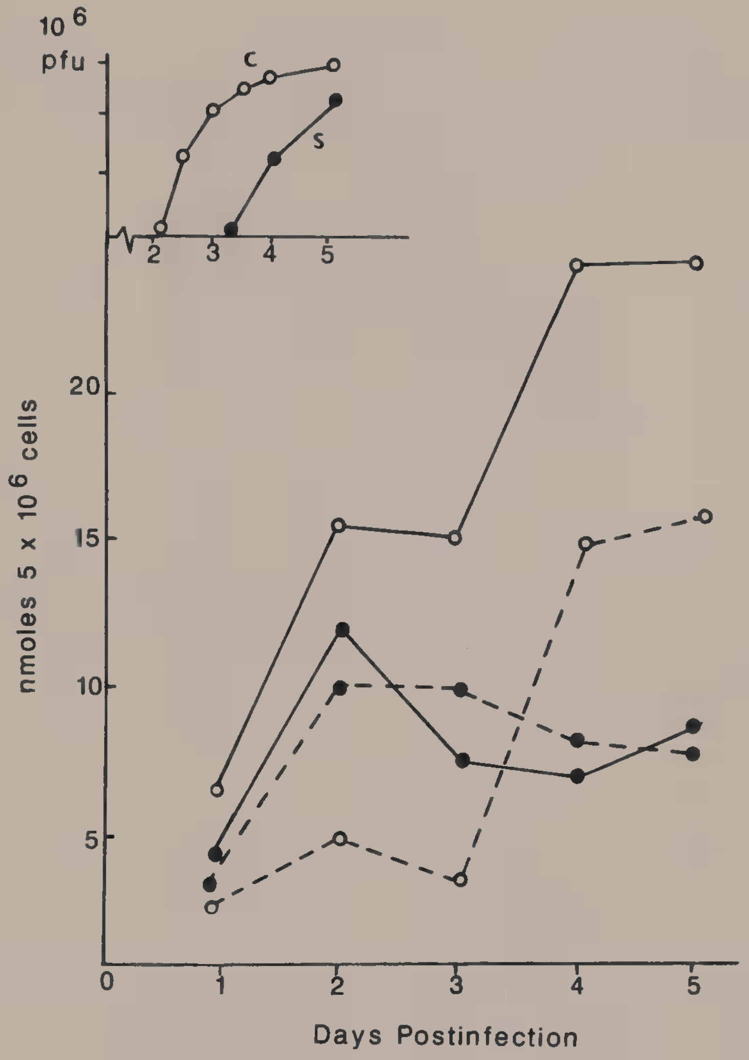

FIGURE 1. Time-course study of polyamine biosynthesis in human embryo fibroblasts infected with CMV. The increase in spermine (○—○) and spermidine (○--○) correlated with the growth of infectivity seen as cell-associated (C) or supernatant (S) virus. In the main figure, the solid symbols represent the uninfected cells. (From Tyms, A. S., Rawal, B. K., Naim, H. M., and Williamson, J. D., *Adv. Polyamine Res.*, 4, 507, 1983.)

fail to code for a novel thymidine kinase activity but induce cellular kinase activities. Unlike human CMV, adenovirus failed to induce ODC after infection of BHK 21, mouse embryo fibroblast, or human embryo kidney cells.[77] The uncoupling of polyamine biosynthesis from other enzyme activities associated with cellular DNA synthesis amounts to a virus-induced modulation of cellular events. It was not clear previously whether or not polyamines were essential factors in adenovirus replication[78,79] but now a role in preserving the integrity of viral nucleic acid appears likely.[80]

The papovaviruses are small DNA-containing viruses also capable of inducing cellular DNA synthesis and the associated enzymes. One member of this group, polyoma virus, induced ODC activity soon after infection.[81] The nature of cellular events at this time appeared to resemble the normal G1 to S phase transition. Polyoma-induced polyamine biosynthesis failed to respond to treatment with 5-fluorodeoxyuridine (FUdR). This was expected as the synthesis of polyamines is normally considered an early event in cell growth. In contrast, the stimulation of ornithine decarboxylase[58] and polyamine biosynthesis[82,83] by CMV infection was prevented by general inhibitors of DNA synthesis such as cytosine arabinoside or FUDR. However, if the DNA inhibitor was highly selective for viral DNA synthesis, no

Table 1
INDUCTION OF POLYAMINE BIOSYNTHESIS BY CLINICAL ISOLATES AND PROTOTYPE STAINS OF HUMAN CMV

	Virus	Patient group	Spermidine	Spermine	Spd:Spm[a]
Clinical isolates	Cx-1	Congenital	40.0	105.8	0.38
	Ha	Renal[b]	10.0	18.4	0.54
	Br	Renal[b]	6.3	16.7	0.38
	FH-2	Marrow[b]	4.6	10.7	0.43
	FH-12	Marrow[b]	27.4	48.3	0.57
Prototype	AD169	nk[c]	12.0	38.6	0.32
	AD169ts13	nk[c]	10.0	19.8	0.50
	Towne	Congenital	4.2	14.4	0.29

Note: Temperature-sensitive mutant kindly provided by Dr. Fred Rapp, Hershey Medical Center, Hershey, Pa. Monolayers of human embryo fibroblasts were infected at a multiplicity of infection of one pfu per cell or greater and cells harvested at 5 days postinfection. The polyamine content was determined by reverse-phase high-pressure liquid chromatography by the method of Clarke and Tyms.[196]

[a] Spd (spermidine):Spm (spermine) ratio in uninfected cells was 1.0 or greater (also see Figure 1).

[b] Transplant patients.

[c] nk = not known.

effect on polyamine metabolism was observed. This selective effect was achieved by the use of 9[1,3-dihydroxypropoxymethyl] guanine (DHPGαnciclovir), an inhibitor of viral DNA polymerase. The compound has potent activity against CMV in vitro,[84,85] with virological and clinical efficacy shown after treatment of life-threatening disease.[86,87] These observations are illustrated in Table 2 by a lack of effect of DHPG treatment on CMV-induced polyamine biosynthesis when compared with the inhibitory action of FUdR. The latter drug inhibits DNA synthesis (virus-specific and virus-induced cellular) by its action on *de novo* nucleotide biosynthesis.[88,89] The initiation of polyamine biosynthesis after CMV infection appears to be closely linked to the induction of host-macromolecular synthesis, in particular the virus-induced synthesis of cell DNA. This may be related to an association of virus-coded enzymes with ODC activity.[58]

C. RNA-Containing Viruses

Bachrach and associates[28] reported that animal RNA viruses, including influenza A and Newcastle disease virus, contained sufficient polyamines to neutralize approximately one third of the genome phosphate groups. Putrescine, spermidine, and spermine have been identified in association with picornaviruses[29,30] and at low levels with the togavirus, Semliki Forest (SF) virus.[90] Although not a human pathogen, SF provides a useful model for the study of the growth of RNA viruses. Tuomi et al.[61] demonstrated that SF virus replication was inhibited by DFMO, although treatment of cells prior to infection was essential. Specificity of the requirement for polyamines was shown by reversal of the block in virus replication by addition of putrescine, spermidine, or spermine or by other polyamine homologues.[61] This suggested that *de novo* synthesis of polyamines was not essential but, as discussed earlier for HSV, levels of polyamines extant within the cells were probably critical for optimal growth. Both ODC and SAMDC activities decreased rapidly after BHK 21 cells were infected with SF virus,[91] supporting this view. The latter study provided evidence that the presence of polyamines in cells infected with SF virus was essential for activity of the virus-coded RNA polymerase. Two major RNA species are synthesized during SF virus

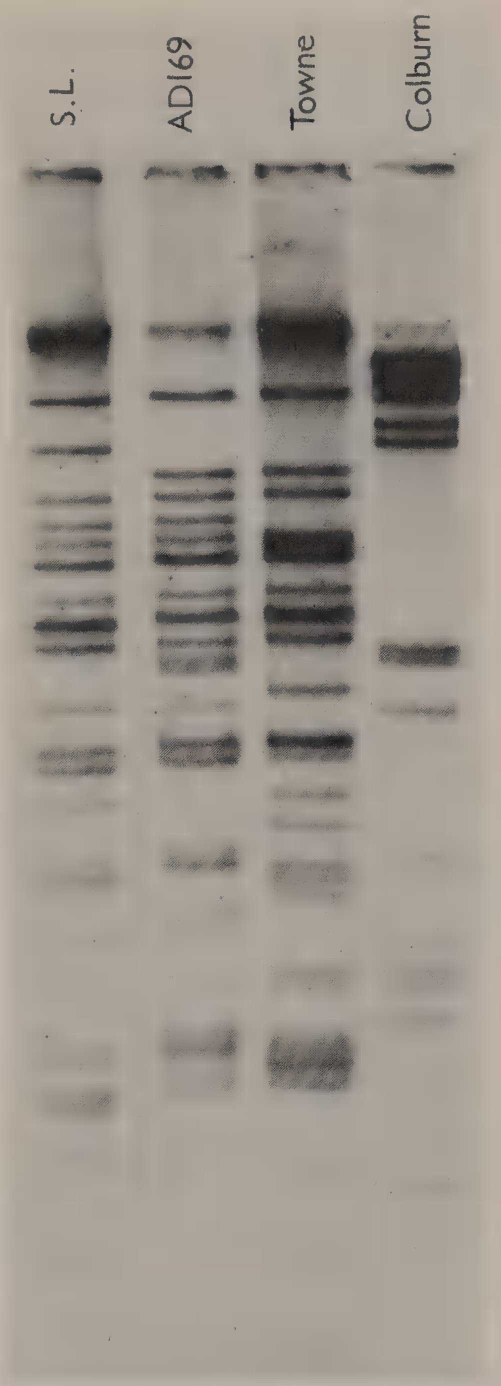

FIGURE 2. Restriction enzyme analysis of viral DNA radiolabeled with ^{32}P orthophosphate and extracted from cells infected with a low-passage clinical isolate (SL), prototype strains AD169 or Towne or the simian-like virus Colburn. DNA was prepared, cleaved with Sma 1 (CCCGGG), and separated by gel electrophoresis as described elsewhere.[68] Extensive comigration of the human isolates conferred identity on the clinical isolate and described the differences in genome structure between human and simian-like strains. This data was kindly provided by Debra Taylor, MCR Collaborative Centre, Mill Hill, London.

replication, a 42S (genome length) and a 26S molecule. Depletion in polyamines prior to infection resulted in a selective inhibition in the synthesis of the 42S RNA species.[92] The smaller molecule was synthesized but provided only the coding sequences for the four structural proteins.[93,94] The effect was rapidly reversed by provision of exogenous polyam-

Table 2
THE EFFECT OF INHIBITORS OF DNA SYNTHESIS ON CMV-INDUCED METABOLISM AT 4 DAYS POSTINFECTION IN HEF CELLS

	Untreated			FUdR			DHPG		
	Un	In	In/Un	Un	In	In/Un	Un	In	In/Un
^{14}C-spermidine	4767	11163	2.3	5894	5125	0.87	6220	19954	3.2
^{14}C-spermine	633	3529	5.6	750	1229	1.6	840	3892	4.6
Spermidine:spermine[b]	1.2	0.8	—	1.4	1.7	—	1.4	0.9	—

Note: The method was according to Clarke and Tyms.[196] Un = uninfected and In = infected.

[a] ^{14}C-spermidine and ^{14}C-spermine were generated after addition of 1,4 ^{14}C-putrescine added 24 hr prior to sampling.

[b] Derived from the total polyamine content.

ines. This selective inhibition of virus-directed events should prove useful in characterizing an important function for polyamines in virus replication.

There is a strong association between polyamine biosynthesis and highly proliferative conditions, in particular cancer (reviewed by Jänne et al.).[95] Polyamine biosynthesis is also linked to virus-induced cellular transformation. A marked increase in ODC and polyamine biosynthesis was associated with transformation of host cells after infection with the RNA-containing oncornaviruses Rous sarcoma[96,97] or murine sarcoma[98] virus. A temperature-sensitive mutant of Rous sarcoma increased polyamine biosynthesis at permissive (37°C) but not at nonpermissive (42°C) temperature.[99] The virus replicated efficiently at both temperatures but the transformed phenotype was evident only at the permissive temperature.

IV. POLYAMINES AND *MYCOPLASMA*

Mycoplasmatales are highly pleomorphic organisms that resemble bacteria but without the rigid cell wall. They can be pathogenic but their importance in experimental studies stems from the fact that they are regular contaminants of cells in culture. Although these organisms can be detected by cytological changes, probably due to mycoplasma-induced nutritional deficiency,[100] most often they go undetected living symbiotically with cells in culture. Up to 25 organisms from the families Mycoplasmataceae and Acholeplasmataceae have been identified as cell contaminants but *M. hyorhinis, M. orale, M. arginini,* and *A. laidlawii* were most commonly found.[101] *Mycoplasma* do not enter the cell but remain associated with the plasma membrane[102] but even so elimination from cell cultures can prove difficult. Methods used include antibiotic treatment,[103] selective killing by incorporation of free base analogues coupled to photosensitivity,[104] or by the use of selective action of macrophages.[105]

The presence of mycoplasma (or bacteria) may cause special problems in the study of polyamines in cell culture due to alternative pathways for putrescine and spermidine biosynthesis. This is further complicated by the lack of information concerning polyamine metabolism in these organisms. Rodwell[106] observed that spermidine and spermine stimulated the growth of certain mycoplasma but only recently has any detailed information on polyamines and mycoplasma become available. In a study of four different strains of mycoplasma,[107] each organism contained high levels of polyamines when grown in cell-free conditions (Table 3). Putrescine, spermidine, and spermine and high levels of cadaverine were measured in two strains isolated from rats and one strain of bovine origin. The detection of spermine was of interest due to the usual absence from prokaryotes and this could be related to the close association of these organisms with eukaryotic cells. In the case of *M.*

Table 3
POLYAMINE CONTENT (NMOL/MG DNA) OF MYCOPLASMAS DURING GROWTH WITH OR WITHOUT DFMO (3 mMOL/ℓ) TREATMENT

Mycoplasma	DFMO	Putrescine	Cadaverine	Spermidine	Spermine
Rat — 2A	−	87	120	1862	27
	+	80	80	1443	28
Rat — 682	−	69	108	1494	54
	+	48	113	1361	48
Bovine	−	78	58	536	10
	+	234	206	694	20
Mycloplasma suispneumoniae	−	1735	nd	nd	nd
	+	3292	nd	nd	nd

Note: nd = none detected. Polyamine levels were determined after 7 days growth in mycoplasma growth medium supplemented with newborn calf serum and/or horse serum.

From Alhonen-Hongisto, L., Veijalainen, P., Kommoner, C. E. K., and Jänne, J., *Biochem. J.*, 202, 267, 1982.

suispneumoniae, putrescine was detected, but not the other three amines. The presence of 3 m*M* DFMO in the growth medium failed to alter the overall pattern of detectable polyamines in all cases. This was evidence that neither ODC nor lysine decarboxylase (LDC) which generates cadaverine, were affected by this potent inhibitor of eukaryotic ODC. Infection of Ehrlich ascites carcinoma cells or L1210 mouse leukemia cells with any of the four organisms resulted in the detection of cadaverine, normally absent from either culture and this was markedly enhanced by DFMO treatment.[107] The synthesis of cadaverine, along with its aminopropyl derivative, was increased in conditions where the cellular putrescine and spermidine content was reduced by DFMO. The synthesis of this diamine may have been a compensatory mechanism for the growth of mycoplasma, which usually depended on a normally abundant eukaryotic source of polyamines. This view is supported by the findings that LDC was expressed by *M. dispar* but ODC was not.[108] Unlike mammalian ODC activities,[109] this enzyme was highly specific for its substrate. The growth of *M. dispar* was totally dependent on the formation of cadaverine as determined by its sensitivity to α difluoromethyllysine which was reversed by the addition of cadaverine. The diamine did not stimulate the growth of *M. dispar* in contrast to the findings of Rodwell.[106]

Evidence that the growth of mycoplasma benefited from a eukaryotic source of polyamines was provided by an association of these organisms with CMV-infected cells. These cells are known to have high levels of polyamines, particularly spermine.[53,71] Darai and coworkers[110] reported that 78% of CMV stocks obtained from laboratories in five different countries were contaminated with mycoplasma. The organisms were identical to the porcine mycoplasma *M. hyorhinis* determined by genome analysis using restriction enzymes.[110,111] Mycoplasma were selectively cultured in mink lung cells (Mv1Lu-ATCC) which were nonpermissive for CMV growth. Such contamination poses special problems in biochemical studies with CMV (or varicella-zoster virus[110]) if the presence of mycoplasma results in coinfection. Nucleoside phosphorylase activity induced by mycoplasma rapidly cleaves exogenously supplied nucleosides to base plus sugar. This prevents the incorporation of uridine or thymidine into nucleic acids by mammalian cells due to their inability, unlike mycoplasma, to utilize free pyrimidine bases.[112,113] For example 90% of thymidine was degraded within 1 hr of mycoplasma-infected mouse 3T3 cells being exposed to the nucleoside.[113] This may cause major problems during experiments if mycoplasma-infected virus stocks are used for DNA analysis. This situation is well illustrated by our own studies.[114] Two species of DNA

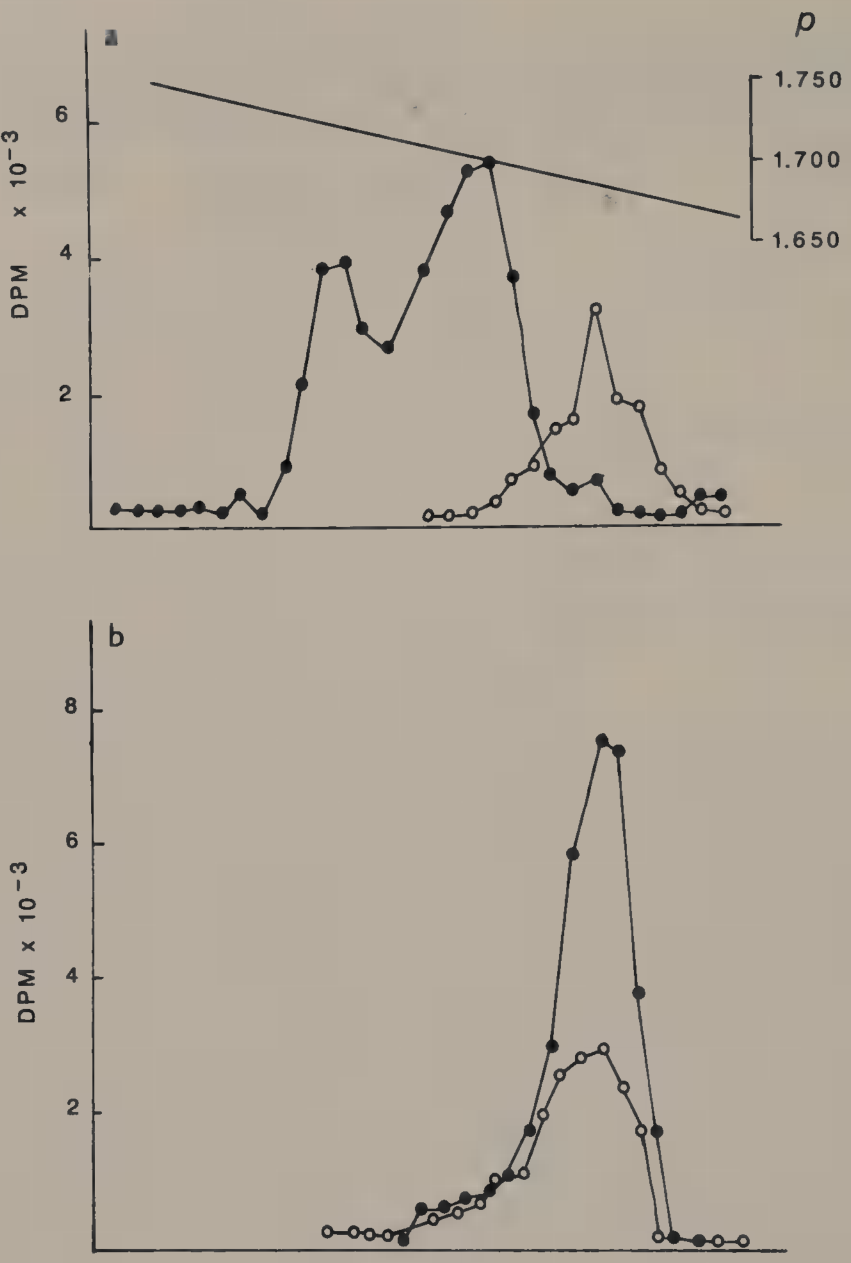

FIGURE 3. Analysis of DNA species radiolabeled with (H-6) thymidine by isopycnic density gradient centrifugation in cesium chloride. Human fibroblast cells were infected with cytomegalovirus stock with (strain Ra) or without (strain AD169) *Mycoplasma hyhorhinis* contamination. Figure a shows comparison of the two strains of cytomegalovirus showing two peaks of DNA for AD169 virus (●—●) corresponding to viral (p = 1.72 g/mℓ) and cell (p = 1.7 g/mℓ). DNA synthesized by strain Ra was in one peak (p = 1.68) which corresponded to mycoplasma DNA (○—○). Figures b and c show the effect of 10 m*M* DFMO treatment (●—●) on the synthesis of mycoplasma DNA and DNA in uninfected control cells stimulated with serum, respectively. (From Tyms, A. S., Taylor, D., and Williamson, J. D., in preparation.)

were synthesized when HEF cells were infected with mycoplasma-free strains of CMV: viral DNA of buoyant density 1.716 g/mℓ and cell DNA of buoyant density 1.699 (Figure 3). Differential analysis was carried out by isopycnic centrifugation using infected cell material previously exposed to [^{3}H-6] thymidine. In the same experiment using a strain of CMV known to be infected with *M. hyorhinis*, radiolabel was only found in DNA of buoyant density 1.682 g/mℓ (Figure 3). Although not radiolabeled, viral DNA was synthesized as identified by restriction enzyme analysis after recovery from the appropriate fractions of the

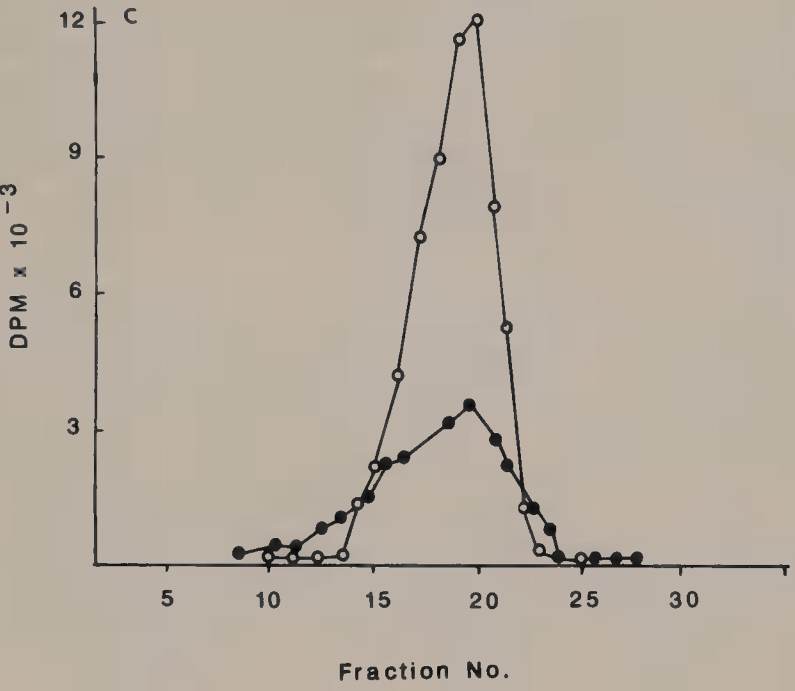

FIGURE 3C

gradient, dialyzed, and ethanol precipitated.[68] This information was consistent with the finding that thymidine was rapidly degraded and selectively utilized for the synthesis of mycoplasma DNA. This illustrates the difficulty in the interpretation of results during DNA analysis but this work also provided information with respect to polyamine biosynthesis. After treatment of serum-stimulated HEF cells with 10 m*M* DFMO, the synthesis of cell DNA (Figure 3) and AD169 DNA (results not shown) was markedly reduced. In cells coinfected with CMV and *M. hyorhinis*, with only mycoplasma DNA radiolabeled, quantitation by density gradient analysis showed an increase rather than a decrease in the amount of mycoplasma DNA synthesized. Although not proven, these results suggest that the growth of *M. hyorhinis* was unaffected by treatment with DFMO and this is consistent with the findings of Alhonen-Hongisto et al.[107]

These discussions serve to illustrate that in vitro studies with mammalian cells or other organisms such as parasites in conditions that favor the growth of mycoplasma, data regarding polyamine biosynthesis as well as nucleic acid metabolism, may be difficult to interpret and could be misleading. Elimination of the organism is difficult if not impossible to achieve but better detection through the presence of cadaverine[107] or the ability to utilize free bases, particularly uracil,[113] or by the use of specific monoclonal antibodies may lessen the likelihood of misinterpretation of results.

V. PHYSIOLOGICAL AND BIOCHEMICAL FUNCTION OF POLYAMINES

During the past 10 to 15 years it has become apparent that polyamines have an essential effect in the various processes involved in cell growth.[63,115,116] Details of the evidence that associates the importance of these molecules with the regulation and synthesis of macromolecules is covered elsewhere in this volume. Determining the true, obligatory nature of polyamine biosynthesis in the growth of eukaryotes in particular has only been possible by the availability of highly specific inhibitors with well-characterized mechanisms of action. DFMO, the catalytically activated, irreversible inhibitor of ODC, has been successfully used to treat serious disease due to trypanosomes[117,118] or *Pneumocystis carinii*.[119] These are major benefits from the clinical application of polyamine research and it is important to understand the critical role of polyamines in the growth of these parasites. Significant clinical benefits from the use of polyamine inhibitors to treat various forms of cancer or disease due to bacteria or viruses have not yet been recorded. Even so, with respect to the present discussions, it is equally important to characterize the function of polyamines in the growth of microorganisms to realize the therapeutic potential of polyamine inhibitors in this area.

A. Bacterial Systems

Estimates of the intracellular concentration of putrescine (0.02 *M*) and spermidine (0.006 *M*) have been determined.[120] Levels of polyamines are directly influenced by extracellular concentration of both amines due to an active transport system in bacteria.[121] Although not normally synthesized by *Escherichia coli*, spermine is also transported into this organism. This could have important implications in the growth and regulation of bacteria in vivo including their survival[122] after the tetraamine is acetylated[123] and further metabolized.

A large volume of literature has been generated by work on macromolecular systems of bacteria in the cell-free state (in vitro tests) and their modulation by polyamines. This has enabled the study of polyamines as regulatory factors in various enzyme systems in nucleic acid and protein synthesis and by an association of polyamines with DNA, for example, using X-ray analysis or nuclear magnetic resonance. However, some of the more interesting observations have come from the study of the whole organism (in vivo tests), particularly by isolation of specific mutants for the various enzymes in the polyamine pathway.

The indication that polyamines were important in protein synthesis through stabilization of bacterial ribosomes was reported a quarter of a century ago,[124] and this view is supported by more recent work. The study of this type of physiological change has necessitated the isolation of polyamine auxotrophs. Due to the complexity of pathways for the synthesis of polyamines, mutants were required which were defective in arginine ureohydrolase, ADC, ODC, and SAMDC. The genetic loci for these enzymes were designated spe A, spe B, spe C, and spe D, respectively.[125,126] Even with *E. coli* strains that had mutations in all four loci, polyamine depletion was nonlethal and the rate of growth was only reduced. Most recently a mutant deficient in spermidine synthase has been designated spe E.[130]

The requirement for exogenous polyamines became absolute when genetic mutants were generated for streptomycin resistance (rps mutation).[127] The rps L mutations affect the S12 ribosomal protein,[128] which could result in an abnormal conformation in the ribosome which would be antagonized by polyamine depletion. An incidental finding was that the antibacterial action of streptomycin by inhibition of protein synthesis was reduced if wild-type bacteria were depleted of polyamines.[128]

Wild-type bacteriophage T7 grew well in the polyamine-deficient mutants of *E. coli* in the absence of exogenous polyamines. However, a decrease in the growth of amber mutants of T7 was observed in such strains of *E. coli* even when the cells were carrying the amber suppressor. The restriction on bacterial growth was bypassed by addition of polyamines to the medium.[131] The putative role of polyamines with the ribosomal complex in translation of mRNA was confirmed in experiments with T7 mutants carrying an amber mutation in gene 1. Here the amber fragment of the protein from this gene was increased in preference to the full-length gene 1 product, providing evidence that polyamines were required for optimal translation of amber codons. One likely defect resulting from polyamine-deficient conditions is in the 30S ribosomal subunit[132,133] due to a decrease in the S1 protein.[134] Evidence for a polyamine requirement in the replication of RNA bacteriophage was also linked to translational events.

Dion and Cohen[135] reported that the T4 phage was unable to grow in polyamine auxotrophs of *E. coli* but this was not confirmed in similar experiments with this phage.[136] Studies of phage in polyamine-deficient conditions, however, suggests this virus is consistently affected by the lack of polyamines, which appear to have an important role in packaging of the genome.[137]

B. Virus-Infected Cells

Polyamine-dependent mutants of mammalian cells have recently been isolated with defects in arginase[138,139] or ODC.[140] Mutant cells such as these should provide interesting host-cell systems for the study of viruses which depend on polyamines, if the wild-type cell is permissive for replication of the virus concerned. This approach to the study of polyamines in virus replication is being applied to selected poxviruses by Williamson at St. Mary's Hospital Medical School.

In earlier studies, the dose response of vaccinia virus to treatment with methylglyoxal bis guanylhydrazone (MGBG), an inhibitor of SAMDC activity,[141] varied in different human cell lines.[53] Virus growth in human embryo fibroblasts (MRC 5) was 100-fold more sensitive to MGBG (90% inhibition at 10 μM) when compared with infected HeLa cells. This may have been due to variation in the transport of MGBG into different cells, although the HeLa cell line is of transformed phenotype and might be expected to accumulate more exogenous polyamines or structurally similar molecules like MGBG. Alternatively, the reported activity of MGBG on mitochondria function[142,143] might predictably induce greater toxicity against heteroploidal cells due to the growth rate than against diploid cells, if toxicity is an adequate explanation for the difference.

Williamson[53] demonstrated that the activity against vaccinia virus was a polyamine-mediated effect by the generation of MGBG-resistant clones of virus. These retained the cell-type associated differences, albeit at correspondingly higher drug concentrations. The work by Hodgson[144] had already provided evidence that SAMDC induced by vaccinia virus infection was a novel enzyme activity and this information coupled with the MGBG-resistant viruses added further support for an obligatory role of polyamines in vaccinia virus growth. The requirement for polyamines is probably mediated late in virus replication because formation of the homogeneous cytoplasmic inclusions, the site of virus maturation, was not observed even though virus-coded macromolecular synthesis appeared unhindered.[52] This agreed with ultrastructural studies that showed morphogenesis of vaccinia virus was dependent on the availability of polyamines.[56] A major function, therefore, of the virion-associated spermidine and spermine[145] appears to be related to conformational changes and packaging of the viral genome but may also be important in the initiation of infection by the virus-associated RNA polymerase.[146]

The proven association of polyamines with intact virions of human herpesviruses[62,147] reinforces the view that the biological cations have an essential role in virion maturation. The membrane-like envelope houses the icosahedral nucleocapsid within which is confined a linear molecule of viral DNA (100 to 150 × 10^6 daltons). Spermine is associated with the nucleocapsid and spermidine with the envelope and intervening tegument region (Figure 4a).

Herpesviruses represent some of the largest mammalian viruses with a coding capacity for a number of proteins with enzymatic activity which are essential for virus replication. Differences in substrate affinities have enabled the successful use of virus-coded enzymes as targets for clinically effective drugs (reviewed by Fiddian et al. in Reference 148). Studies with viral enzymes, particularly those associated with DNA metabolism, in cell-free systems suggest that modulation of essential enzyme functions may be possible by suboptimal concentrations of spermidine or spermine. The DNA polymerase activities of HSV type 1 and type 2 showed a requirement for polyamines at concentrations in the physiological range when analysis was carried out in cell-free conditions.[149-151] In total cell-lysates of BHK 21 cells infected with HSV type 1, genome length molecules were synthesized whereas in the polyamine-permeable nuclear preparations of the same infected cells, viral DNA was

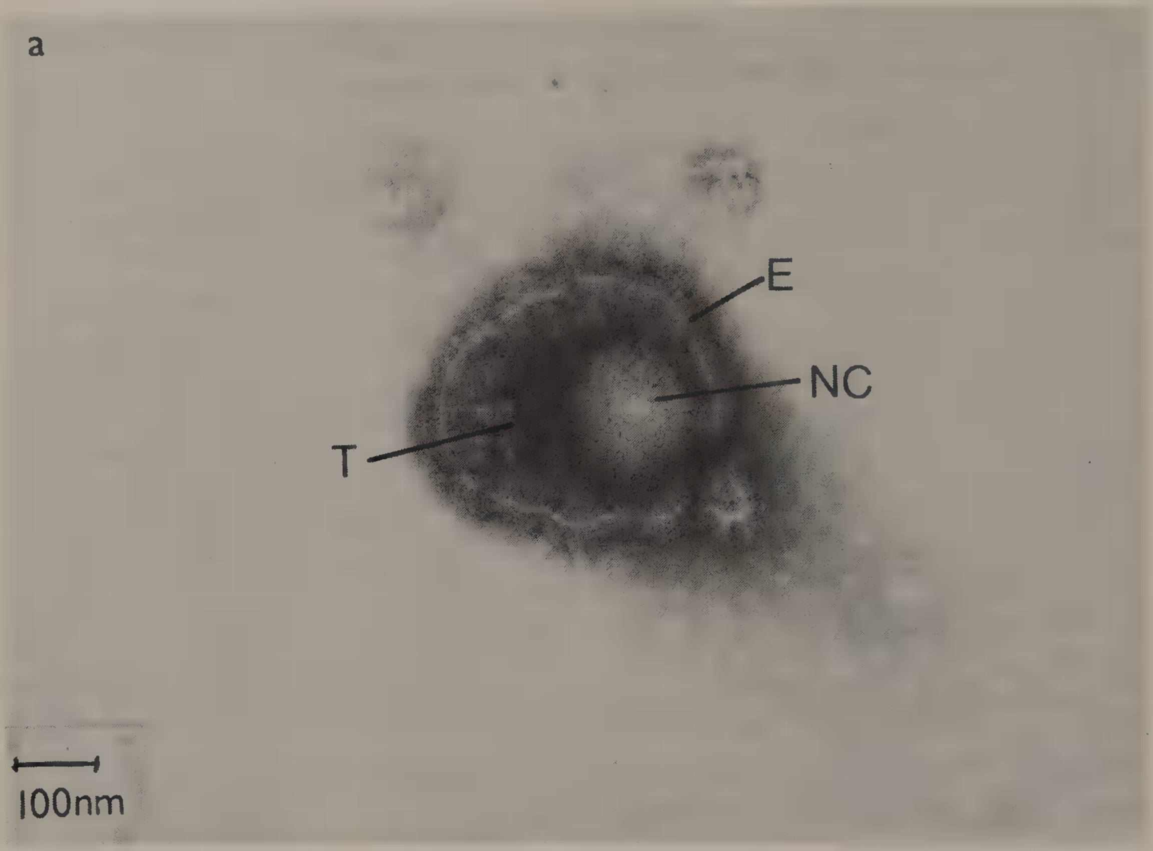

FIGURE 4. (a) Electron micrograph of a human CMV virion in the mature state negatively stained by phosphotungstic acid. The nucleocapsid (NC) is surrounded by the tegument region (T) and envelope (E). (b) Electron micrograph of an ultrathin section prepared from the kidney of a baby that died of disseminated herpes simplex infection. Groups of nucleocapsids (NC) are visible along with the characteristic margination of chromatin (Ch). (Presented by kind permission of Tim Ryder and Margaret Mobberley, Electron Microscope Unit, Queen Charlotte's Hospital for Women, London.)

subjected to extreme endo- and exonuclease activity.[152] By adjusting the polyamine levels close to the novel intracellular concentration the nuclease activity was inhibited. The apparent protective role of polyamines against nuclease activity may relate specifically to the viral genome rather than cellular DNA, thus providing a selective protection for viral DNA.[150] Endonuclease and exonuclease activity of the viral DNAase of both HSV[153] may well be related to the redistribution and damage to chromatin after infection (Figure 4b). Both enzyme activities respond to polyamines when tested in vitro but, as in bacterial systems, such in vitro investigations need to be confirmed by in vivo studies. Experiments with adenovirus type 5 infections of cultured cells have provided evidence that this protective mechanism with respect to nuclease activity exists in vivo. The degradation of spermine mediated by polyamine oxidase after adenovirus infection, which resulted in extensive chromosomal damage, was prevented by increasing the exogenous polyamine levels. Furthermore, treatment with the inhibitor of polyamine oxidase, aminoguanidine, provided similar protection for cellular DNA.[154]

The evidence available from the cell culture systems infected with HSVs or adenoviruses points to a virus-mediated block in the *de novo* synthesis of polyamines.[58,60,77] Only by depletion of polyamines in cells prior to and during infection with HSVs with DFMO[61,62] or MGBG[53] did virus growth respond to the polyamine inhibitors. As discussed earlier, human CMVs consistently induced the synthesis of polyamines with a marked increase in spermine levels (see Figure 1 and Table 2). The obligatory nature of this biosynthetic activity was deduced from the sensitivity of virus growth to treatment with polyamine inhibitors. Prior treatment of cells, particularly with DFMO, may be essential to gain maximum antiviral effect.[63]

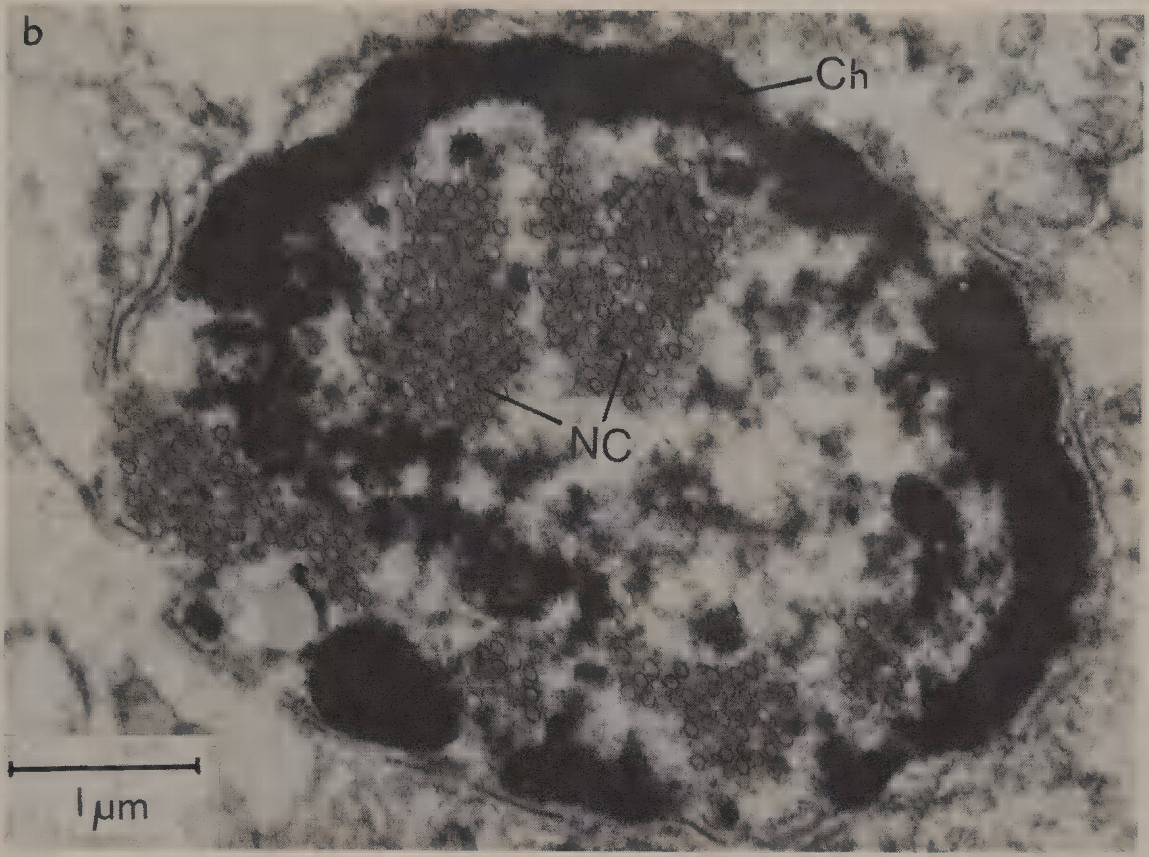

FIGURE 4b

Other analogues of ornithine or putrescine have been synthesized which are also catalytically activated, irreversible inhibitors of ODC.[117] α-Monofluoromethyldehydroornithine methyl ester (Δ-MFMO-methyl ester) had 10 to 12 times greater activity against ODC than the parent compound Δ-MFMO or DFMO. The putrescine and spermidine levels in suspension cultures of tat hepatoma cells (HTC) was markedly reduced by DFMO, Δ-MFMO, or Δ-MFMO-methyl ester but at one tenth the concentration in the case of the latter compound.[156] With all three compounds, the level of spermine remained unchanged and SAMDC activity was increased presumably in response to the spermidine depletion. Treatment with Δ-MFMO-methyl ester (1 m*M*) completely blocked the growth of CMV.[5,157] The drug concentration corresponded to about one tenth that required for DFMO to inhibit this virus,[62,155] and the differences in dosage between the two drugs was consistent with the studies cited above.[156] The most profound effect of Δ-MFMO-methyl ester on CMV growth was accompanied by an inhibition in the synthesis of viral DNA and consequently the expression of the abundant late proteins.[5] This correlated with the marked depletion in spermidine levels while spermine, in common with the studies of Mamont et al.[156] remained at levels similar to uninfected, quiescent cells. The high spermine levels may in part be a result of the increased SAMDC activity seen after Δ-MFMO-methyl ester treatment[156] or due to raised levels of polyamine synthase activities induced by CMV.[159]

In terms of the mechanism of antiviral action of polyamine depletion, it is likely that more than one essential virus-coded or host-coded function is involved. Gibson et al.[62] concluded from their studies that polyamine depletion had little effect on the synthesis of the major viral macromolecules but that encapsidation of the viral genome or the acquisition of its envelope may be susceptible. In view of the abundance of negatively charged components of the virion, DNA, phosphoproteins, and phospholipids, it would make sense to associate these biocations with a major role in virus maturation.

In our own studies, different human fibroblast cell strains infected with human CMV have responded differently to treatment with DFMO. For example, estimates of an effective inhibition by DFMO using plaque reduction assay, ranged from 10 to 15 m*M*.[5] This procedure exposes cells to treatment with the compound prior to infection in much the same way as the protocol described by Gibson et al.[62] In certain fibroblast lines DFMO (10 m*M*) was totally inhibitory after addition at the time of infection[155] and this involved a stage prior to the synthesis of viral DNA. This early effect is most often observed with the high doses of Δ-MFMO-methyl ester and referred to above, although it is now becoming clear that less stringent inhibition of polyamine biosynthesis with lower concentrations of this compound modulates virus growth at a later stage. This may involve virion maturation probably through an effect on the synthesis of late structural proteins. Both the 69-kdalton matrix protein and 53-kdalton DNA-binding protein are expressed at low levels early in virus growth[158] and amplified at a later stage. Both proteins were particularly susceptible to treatment with Δ-MFMO-methyl ester.

VI. CLINICAL APPLICATION OF POLYAMINE BIOSYNTHESIS IN MICROBIOLOGY

The enormous research effort that has been applied to the investigation of polyamines over the last few years both in and out of the medical environment is now beginning to show benefits in terms of clinical application. The discovery of DFMO, now known as eflornithine monohydrochloride, and its ability to cure experimental trypanosomiasis as well as African sleeping sickness in man provided the first step in achieving this aim. Activity of this compound has been shown against other parasitic infections including *Plasmodium* sp., *Leishmania* sp., *Eimeria tenella, Giardia lambia,* and most notably the compound has found application in the treatment of *Pneumocystis carinii* pneumonia and Cryptosporidia infections in AIDS patients.[117,119,160] This area of research is covered elsewhere in this book. In terms of virus infections of man, and to a lesser extent bacterial infections, two areas of clinical interest have emerged during the last few years: the possible application of polyamine biosynthesis as antiviral or antibacterial agents and the use of polyamines or their derivatives as markers for infection and disease.

A. Polyamine Inhibitors as Antimicrobial Agents

The problems related to providing drugs with potent antiviral effect while maintaining a wide therapeutic index have been difficult to overcome. This is due to the necessity of a viable host cell for the replication of animal viruses. With human herpesviruses this has been achieved through the exploitation of virus-coded enzymes as novel targets for drug action, work pioneered through the development of acyclovir [9-(2-hydroxyethoxymethyl) guanine].[148] There is little or no utilization of this compound in uninfected cells due to the lack of a virus-coded kinase to initiate phosphorylation and no virus-coded DNA polymerase for activity of the phosphorylated form during the synthesis of polynucleotides. Other compounds with a different mode of action are needed as alternatives and also to complement the drugs presently available.

Little is known about the nature of the enzymes when the polyamine pathway is induced by virus infection. Enzyme characterization, particularly the decarboxylases, will be a difficult task although provisional studies with vaccinia virus suggests that ODC and possibly SAMDC may differ from the normal activities expressed in the uninfected cells.[51,144] Quantitative as well as qualitative differences in enzyme activities induced by virus infection may be open to exploitation if the function of the pathway is obligatory for virus growth. Interestingly, the selective action of DFMO in the treatment of trypanosomal infections has, at present, no clear scientific explanation. DFMO has similar inhibitory characteristics against trypanosomal and mammalian ODC, and its entry into the cell is not facilitated in either case. The rapid rate of growth of the parasite and the need for polyamines during differentiation are almost certainly major factors. These, coupled to a reliance on spermidine as the sole polyamine component, may be adequate to explain the remarkable selective action of this drug. The presence of a competent immune system is also a necessity,[161] and this is likely to be of major importance in the treatment of viral infections.

Human CMV and vaccinia virus are both capable of inducing disease in man and known to depend on the synthesis of polyamines for successful replication. CMV is most important as a cause of severe disease in the newborn[162] and serious life-threatening disease in the immunocompromised host.[163] Most recently, this virus has emerged as a major cause of morbidity and mortality in patients with AIDS.[164] Also, the possible reintroduction of vaccination as a measure to control other disease using vaccinia as a vector[165] may demand a means of treating possible post-vaccination illness.

The sensitivity clinical isolates of CMV to MGBG, a potent inhibitor of SAMDC,[141] was much greater than that recorded for the known inhibitors of herpesvirus replication, 5-iodouridinedeoxyribose (IUDR) or acyclovir.[155] MGBG was first used in cancer chemotherapy more than 20 years ago[166] although it was constantly associated with side effects after treatment. Mitochondrial damage was observed in L1210 leukemia cells when grown in vitro[142] and in vivo,[167] but this only occurred when MGBG became concentrated to millimolar levels during high rates of cell division.[143] The antiviral effect observed against human CMV in vitro was not seen with HSV unless HEF cells were pretreated prior to infection. Similar observations were made in mouse embryonic fibroblasts during comparison of MGBG-sensitive murine CMV. In common with the human strains, the murine virus is known to induce polyamine biosynthesis which is not the case for HSV.[53] This was evidence for selective action against CMV-induced events without damage to the host cell but more convincing evidence would come from the study of drug-resistant mutants. Such mutants have been selected for in the case of vaccinia virus.[53] The ED50 for MGBG against vaccinia was 3.5 μM in HEF cells, whereas MGBG-resistant strains were about 100-fold less responsive to the drug than the wild-type virus.

There has been renewed interest in the potential of MGBG in the treatment of cancer through a better understanding of dose-dependent effects. In the treatment of childhood leukemia, the therapeutic response to MGBG, albeit temporary, showed a ‘‘therapeutic index’’ could be achieved by selective accumulation of the drug in circulating blast cells.[168] The antitumor effect was enhanced by priming the patients with DFMO treatment prior to administration of MGBG, although there is evidence that this approach to therapy may also enhance MGBG uptake in nontumor tissue.[169] The selective uptake of polyamine-like molecules by metabolically active cells is attractive in the case of CMV. This virus is known to induce the host cell into high metabolic activity after infection.[58] The potential toxicity of MGBG, however, has excluded its clinical application for CMV infections. Work with MGBG has illustrated the antiviral properties of spermidine analogues and these may have potential value for future exploitation. Porter and colleagues[170] have investigated a series of N^4 and N^1- N^8 spermidine analogues against the rapidly dividing L1210 leukemia cells in culture for their ability to suppress decarboxylase activity. N^1-N^8-bis 4(ethyl)spermidine (BES) was the most effective with no activity when substitutions were made in the N^4 position. BES was active against both ODC and SAMDC but the novel regulatory mechanism operative against ODC was of most interest and provided a new concept in polyamine inhibitors.

B. Antiviral Activity

The clinical efficacy of DFMO in the treatment of parasitic infections with apparently little or no side effects provided most encouragement when considering the possible application of polyamine inhibitors against viral disease. The antiviral activity of DFMO against CMV infections in vitro is well established[5,62,155] although its exact antiviral mechanism has not been defined. Direct clinical studies of DFMO against CMV-induced disease have not been initiated but indirect observations have been made when the drug was administered for other purposes. Golden, Sjoerdsma, and Santi[119] described the first successful use of DFMO in patients with acquired immune deficiency syndrome (AIDS) affected by *Pneumocystis carinii* pneumonia which was unresponsive to conventional therapy. The parasitic disease is often accompanied by severe opportunist infections with human CMV which can include neurological complications.[171] Six patients with *P. carinii* pneumonia responded to DFMO treatment using a protocol of 4 to 6 g/m^2 body surface per day orally for 6 to 7 weeks which produced little or no toxicity.[119] In two out of six patients, CMV was cultured from the lung before and after treatment and one patient, although negative for CMV at the completion of treatment eventually died of disseminated CMV disease. In the remaining three patients, CMV infection was not diagnosed. Even though a much greater number of AIDS patients have now been treated successfully for *P. carinii* pneumonia, there is no firm indication of a response of CMV infection to the drug. The study of potential antiviral activity of DFMO in these patients is made difficult by the nature of the syndrome especially as an intact immune system is known to be vital for the antiparasitic activity of the drug.[161]

A well-recognized problem with DFMO is its poor uptake into mammalian cells. This problem may be circumvented by the use of prodrugs and this approach to therapy has already been addressed[117] and discussed earlier in terms of increased effect against polyamine biosynthesis. Facilitated uptake of Δ-MFMO-methyl ester with activity in the form of the parent compound after cleavage by nonspecific esterase activity was the basis of the increased potency.[156] Administration of the drug to rats (25 mg/kg/day) decreased ODC by 80% and putrescine or spermidine by 90% in the ventral prostate.[156] Most important, Δ-MFMO-methyl ester treatment produced better protection in mice infected with *Plasmodium berghei* through inhibition of exoerythrocytic schizogony[172] and had greater curative effect in murine trypanosomiasis, than DMFO.[173] More information is required on the possible use of this compound or other prodrug forms of ODC inhibitors in experimental systems, particularly in regard to toxicity. The fact that Δ-MFMO-methyl ester is a potent inhibitor of CMV growth with better antiviral activity than DFMO is encouraging with respect to future investigations in the treatment of viral disease.

C. Other Considerations

Earlier reference was made to the use of MGBG to treat childhood leukemia, both alone and in combination with DFMO.[168] The principle here was a depletion in polyamine levels induced by priming with the ODC inhibitor enhanced the uptake of the spermidine analogue into circulating blast cells. Similarly, depletion of polyamines in an experimental mouse model of trypanosomiasis increased the efficiency with which the bleomycins A5 and A6 cured semichronic infections.[174] These antibiotics have spermidine and spermine, respectively, as terminal amines and hence binding to and scission of DNA is enhanced in conditions of polyamine depletion.[175] The combined use of DFMO with interferons or cytotoxic drugs[176-178] may well provide a major role for DFMO or its analogues in cancer chemotherapy.

Drug combinations using well-defined but preferably distinct targets may offer potential in antiviral chemotherapy. Polyamine levels appear to be critical for the function of virus-specific enzymes[149-151] and to maintain the integrity of host (and possibly viral DNA) with respect to nuclease activity.[153,154] DFMO treatment is known to modulate the replication of human CMV[62,155] and HSV ,[61,62] and the combination of polyamine inhibitors with the potent antivirals, DHPG and acyclovir, is clearly made possible by the clinical use of all three drugs. Combined use in vitro of acyclovir and DFMO against HSV appeared not to increase the singular drug effect.[5] However, preliminary data suggest a synergistic or additive effect when human CMV was treated with a combination of DHPG with DFMO[179] or ΔMFMO methyl ester.[180] The inhibition of polyamine biosynthesis after infection with HSV[53,59] suggests that polyamine inhibitors can only exert effect by depleting existing levels of these substances. There is some evidence, however, that the degradative pathway which generates putrescine and spermidine from higher polyamines by acetylation and diamine or polyamine oxidase activity may operate in the case of HSV.[4] Inhibitors of oxidase activities may therefore be of interest experimentally in combination with drugs already in clinical use, such as acyclovir.

Other enzymes in the anabolic pathway offer potential targets for inhibitors and this is particularly so for human CMV. A three- or fourfold increase in measurable spermidine and spermine synthase activity was observed after virus infection[159] and this would help account for the marked increase in spermine levels in virus-infected cells. Spermidine synthase alone is sensitive to inhibition by the transition state analogue *S*-adenosyl-1,8-diamino-3-thiooctane[181] or by exposure to dicyclohexylammonium sulfate.[182] A derivative of MGBG, methylglyoxalbis(butylamidinohydrazone) (MGBB) also inhibited spermidine but not spermine synthase and was a powerful inhibitor of both decarboxylase activities.[183] Once again the potential toxicity of such drugs needs close attention but multitargeted drugs may be exploitable if a significant chemotherapeutic index can be derived by dose-dependent differences in their effect against virus as opposed to the host-coded events.

D. Antibacterial Agents

In the genetic studies referred to earlier, it was apparent that the growth of auxotrophs with defects in the four loci responsible for expression of the major enzymes in prokaryotic pathway, bacterial growth was incompletely blocked. Even so, the evidence is much in favor of a major role for polyamines to provide optimal conditions for the growth of bacteria. The pitfalls involved in constructing genetic mutants with total reliance on exogenous polyamines reflects on the equally difficult task of finding a means to inhibit the metabolic activity in bacteria. This could involve compounds specific for the various enzymes for use in combination or compounds with activity against a number of enzymatic steps as indicated above with MGBB.

Although little work has been carried out with polyamine inhibitors as potential antibacterial agents, there is evidence that this is feasible with some bacteria. Both the biosynthetic and degradative ODC activities extracted from *Pseudomonas aeruginosa* were sensitive to inhibition by DFMO but this was not the case for *Escherichia coli* or *Klebsiella pneumoniae*.[184,185] Conversely the biosynthetic ODC activity from *P. aeruginosa* only was inhibited by α-monofluoromethyl-putrescine while both ODC activities were sensitive to α-monofluoromethylornithine.[185] This effective inhibition of ODC was not sufficient to reduce putrescine levels due to the compensating mechanisms of ADC.[184] A reduction in polyamine level and growth rate of both *P. aeruginosa* and *E. coli* was achieved only when, in addition to the block in ODC activity, ADC and spermidine synthase activities were also inhibited. This was achieved with α-difluoromethyl-arginine and dicyclohexyl-ammonium sulfate, respectively.[18,185] The inhibition of spermidine synthase was essential to complete the depletion of polyamines due to the increase in spermidine levels when a combination of both decarboxylase inhibitors reduced the synthesis of putrescine.

These studies serve to illustrate a number of major problems when considering the possible use of polyamine inhibitors for antibacterial therapy. First, the existence of multiple pathways for the generation of polyamines; second, the fact that depletion of both putrescine and spermidine is essential; and third, that bacterial enzymes tend to vary in their sensitivity to different inhibitors. Obviously the ideal concept of a single enzyme target with single antimetabolite which is the case with the antiparasitic work, does not apply. It is possible that compounds with broad-spectrum activity against a number of steps in polyamine biosynthesis may have an advantage as antibacterials. Likewise, the possibility of combination therapy with unrelated drugs in current use may be worthy of consideration.

E. Polyamines as Clinical Markers for Microbial Infections

Polyamine biosynthesis has long been known to be important for cell growth and differentiation[63] and now of major significance is the clear association of altered polyamine metabolism with cell proliferative disease. Russell and colleagues[186] first provided evidence that patients with cancer had elevated levels of polyamines in their urine. Since then numerous studies have reinforced the observation of aberrant levels of polyamines[187-189] or their acetylated derivatives[190,191] in body fluids or secretions which may provide markers for use as a rapid screening test for the detection of malignancy.[192]

In our own studies, CMV is characterized by long-term infections in cell culture which is accompanied by continuous synthesis of viral proteins, nucleic acid, and polyamines.[71]

This appeared to mimic the long-term viruria observed after congenital or postnatal infections.[162] Worldwide estimates of the incidence of congenital infection is between 0.3 and 3.5% of births,[193] but only about 10 to 20% of these undergo symptomatic infections. A proportion of the asymptomatic group eventually suffer from more subtle effects of CMV infection, e.g., sensorineural deafness.[194] The virus persists in numerous tissues and probably cells of leukocyte lineage.[195] It was possible that the high-titer viruria, often present for 3 to 4 years of early childhood, might be reflected by altered polyamine metabolism and excretion.

Isolation and identification of CMV can be difficult and time consuming and because of the high proportion of asymptomatic cases, diagnosis is not attempted in most instances. The clandestine nature of CMV infection after congenital or acquired infection coupled to the potential hazard of virus excretion to women of childbearing age, would make rapid screening tests extremely valuable. A clinical chemistry procedure would be a major asset in any screening program for infection and disease.

Preliminary results from our studies showed that elevated levels of spermine may provide a marker for CMV infection.[70]

Of 74 urines from babies culture positive for CMV after congenital or postnatal infection, 59 (80%) had detectable levels of spermine with a mean value of 12.4 nmol/mg creatinine (+/− 4.6 nmol/mg). Of 100 urines from a control group of babies who were negative for CMV, 80 had no detectable spermine measured by reverse-phase HPLC.[70] The lower level of sensitivity with this method was 200 pmol/mℓ sample.

In those urines with detectable spermine, the mean value was 6.4 nmol/mg creatinine (+/− 4 nmol/mg. The CMV-positive group included two babies hospitalized with symptomatic infection and an association was established between spermine levels, virus excretion, and symptoms of disease. There was no clear correlation between CMV infection and putrescine or spermidine levels, but spermine was considered of major significance for three reasons.

1. High spermine levels predominate after CMV infection in vitro.
2. Spermine was not readily detected in CMV negative babies by our reverse-phase HPLC method.[196]
3. Spermine is not usually synthesized by bacteria, which are a major problem in terms of contamination of urines in young babies.

Studies are in progress to examine for the acetylated derivatives of polyamines, in particular *N*-acetylated spermine, as this polyamine derivative appears to be infrequently found as an excretion product. It will be important to investigate patients with CMV disease for altered polyamine excretion both before and after antiviral chemotherapy with DHPG. This drug has a major effect on the excretion of virus[87] and its presence in lung biopsies[86] from patients with disease due to CMV. Due to its specificity for cells infected with herpesviruses, modification in the pattern of polyamine excretion after treatment would provide confirmation of the virus association.

The amine content of vaginal washings from patients with nonspecific vaginitis (NSV) can be used as a marker for this condition.[197] Putrescine and cadaverine were most abundant in samples from women with NSV but absent from nonaffected individuals.[197] The presence of diamines did not distinguish between NSV and vaginitis caused by infection with the parasite, *Trichomonas vaginitis*.[197] Both conditions, however, responded to treatment with metronidazole which resulted in a corresponding loss of detectable amines.[197,198] Although the bacterial etiology of NSV is uncertain at present,[199] the clear association of putrescine and cadaverine with the condition is important in diagnosis. The availability of rapid analytical procedures with a high level of sensitivity[192] could be of major benefit if applied to clinical microbiology.

VII. CONCLUDING REMARKS

An important step in scientific or medical research is when the topic under investigation is shown to be of major benefit in clinical terms. This has been achieved through polyamine research by the proven clinical efficacy of ODC inhibitors in the treatment of parasitic infections. This success not only provided unequivocal evidence for the obligatory nature of polyamines during reproduction but also provided essential toxicology data on the clinical use of DFMO. This information, along with exact knowledge of the mechanisms involved in the inhibition of growth of parasites, will be extremely important when considering the application of this therapeutic strategy to other microbial disease.

Other factors will influence the approach to therapy with polyamine inhibitors. Evidence for the polyamine pathway being a cyclic process may complicate the issue although the acetylation and oxidative reactions involved in the degradative pathway do offer novel targets for new drugs to complement those presently available. This may of particular importance with bacteria when considering the apparent difficulties in attempting to completely suppress the growth of these organisms by treatment with polyamine inhibitors. Already there is preliminary evidence that the catabolic process is important to satisfy the polyamine requirement during the replication of HSV.

The catabolic pathway is known to generate oxidation products that act as inhibitory substances on cell growth and appear to have important regulatory properties on the immune system. Low concentrations of spermidine or spermine-inhibited in vitro lymphocyte proliferation provided polyamine oxidase activity in the form of bovine serum was present.[6] It has been postulated that modulation of the immune response in AIDS patients may be exacerbated by repeated contact with semen or infection with CMV,[200] both of which can be related to increased polyamine levels in vivo.

The discovery and application of new drugs that inhibit single or multiple enzymes in the polyamine pathway should enable further exploitation of the pathway provided toxicity can be avoided. The prodrug α-monofluoromethyldehydroornithine methyl ester has greater potency than DFMO against virus and parasitic infections. It is also clear that spermidine analogues could be important in treating disease particularly if selective uptake as demonstrated for MGBG is possible. Recent studies are providing a better understanding of polyamine transport in animal cells. Certain tissues are known to accumulate polyamines by energy-dependent transport systems. Slices of lung, seminal vesicle, and brain cortex accumulated spermidine or MGBG by this mechanism but this was not the case for heart, liver, spleen, or kidney cortex.[201] More detailed studies of lung tissue have shown that the active transport mechanism is restricted in the main to type I and type II pneumocytes.[202]

The lung is a prime target for many virus infections, including human CMV, and the uptake of polyamine inhibitions selectively into infected cells is an attractive proposition.

An essential role for polyamines in the growth of bacteria and viruses is undisputed but the exact details of their function is not fully understood. In bacteria, efficiency of transcription and translation have long been known to depend on optimal concentrations of polyamines. More recently, studies with virus-infected cells have also shown an important role in the expression of viral genes. Only partial transcription of the SF virus genome occurred during polyamine depletion and this was probably mediated at the RNA polymerase level. The efficiency of initiation of transcription by the virion-associated RNA polymerase was reduced during the growth of vaccinia in polyamine-depleted conditions. An effect on transcription appeared to be one consequence of polyamine depletion in CMV-infected cells. The clear association of the biological cations in packaging of the viral genome and the putative role in enzyme function in terms of both synthetic and degradative function also warrant further investigation as potential targets for chemotherapy.

Considerable progress has been made in the utilization of polyamines as markers for cancer, particularly in terms of response to chemotherapy. The recent advances in understanding the complexities of polyamine metabolism and the variety of polyamines now being identified in bacteria could enable greater application of these substances as specific markers in disease. The work with CMV has already indicated potential benefits in an area of diagnostic virology where a rapid test for active virus growth would be invaluable. Sensitive methods for the detection of polyamines and their derivatives now becoming available for clinical use in the diagnosis of cancer may soon be applied routinely in microbiology departments.

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Chapter 2

POLYAMINES IN THERMOPHILES

Tairo Oshima

TABLE OF CONTENTS

I. Thermophiles 36

II. Polyamines in Thermophilic Eubacteria 36

III. Polyamines in Archaebacteria 39

IV. Chemistry of Unusual Polyamines 39

V. Biochemistry of Polyamines in Thermophiles 42

References 44

I. THERMOPHILES

Thermophiles are fascinating organisms for biochemists and molecular biologists.[1] Biopolymers from thermophiles are, in general, unusually stable to heat. The heat-stable enzymes are of special interest in enzyme engineering and protein engineering[2] as well as in protein chemistry. Besides proteins and nucleic acids, some of the cell components in thermophiles are also unusual; for example, a unique modified base, 2-thioribothymidine, which is present in the T loop, is essential for the unusual thermostability of tRNA from an extreme thermophile.[3] Membrane lipids of thermophilic eubacteria and archaebacteria are different from those of mesophilic organisms.[4]

Though there is no clear borderline, usually organisms which are capable of growing at 55°C or higher, are called thermophiles. The organisms which are capable of growing at temperatures higher than 75°C are classified as extreme thermophiles; others are moderate thermophiles. Among moderate thermophiles, *Bacillus stearothermophilus* has been studied most extensively so far, and is a spore-forming, rod-shaped bacterium. This microorganism is widely distributed in nature and has been isolated from soil, compost, sludge, hot springs, and even nonthermal environments such as river water or mud from the ocean bottom. Thermophilic anaerobic bacilli have been known for many years. One example is *Clostridium thermohydrosulfuricum* which grows over a temperature range of 40 to 78°C with production of ethanol.

The best studied extreme thermophiles are those belonging to the genus *Thermus,* such as *T. thermophilus, T. aquaticus*, and *T. flavus*; they are Gram-negative, nonspore-forming, pigmented rods, inhabiting hot springs. The highest growth temperature for these bacteria is about 85°C. The polyamine composition of *T. thermophilus* has been extensively studied, as described in the next section.

Some archaebacteria[5] are thermophilic.[1] Thermophilic methanogens, such as *Methanobacterium thermoautotrophicum,* have been known.[5] *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum* are acido-thermophilic archaebacteria; the former optimally multiplies at pH 3.0, 75 to 85°C, and the latter at pH 1.5 to 2.0, 58°C.

The thermophiles capable of growing at temperatures higher than 85°C so far known are classified into archaebacteria.[6] *Thermoproteus tenax, Desulfurococcus mobilis*, and some other archaebacteria grow optimally at 85°C under anaerobic conditions in the presence of elementary sulfur as electron acceptor. *Pyrodictium brockii* holds the record of growth temperature; this autotrophic archaebacterium grows at 110°C in the presence of H_2 and sulfur under anaerobic condition. Unfortunately only a few studies have been done on their biochemical properties and nothing has been reported for their polyamine compositions.

II. POLYAMINES IN THERMOPHILIC EUBACTERIA

In general, mesophilic bacteria contain no spermine[7,8] or other tetraamine. However, thermophilic bacteria contain tetraamines. In a moderate thermophile, *B. stearothermophilus,* spermine was found along with putrescine and spermidine.[9] Likewise an acido-thermophilic eubacteria, *B. acidocaldarius* contains spermine, spermidine, and putrescine. Anaerobic thermophile also contains spermine. It can be said that thermophilic eubacteria generally produce spermine and/or other tetraamine though the presence of spermine in some mesophilic bacteria has also been described.[7]

An extreme thermophile, *Thermus thermophilus*, is the most interesting organism in the view of chemistry and biochemistry of novel polyamines. At least 14 polyamines have been detected in the cells of this extreme thermophile and another has been suggested to be present in the cells. These 15 polyamines are listed in Table 1. Among them, ten amines have been isolated and purified from the cell extracts, and their chemical structures were confirmed

Table 1
POLYAMINES IN THE CELLS OF AN EXTREME THERMOPHILE, *THERMUS THERMOPHILUS*

Trivial name	Systematic name	Chemical structure	Ref.
1,3-Diaminopropane	1,3-Diaminopropane	$NH_2(CH_2)_3NH_2$	
Putrescine	1,4-Diaminobutane	$NH_2(CH_2)_4NH_2$	
Norspermidine (= caldine)	1,7-Diamino-4-azaheptane	$NH_2(CH_2)_3NH(CH_2)_3NH_2$	10, 11
Spermidine	1,8-Diamino-4-azaoctane	$NH_2(CH_2)_3NH(CH_2)_4NH_2$	11
sym-Homospermidine	1,9-Diamino-5-azanonane	$NH_2(CH_2)_4NH(CH_2)_4NH_2$	11
Thermine	1,11-Diamino-4,8-diazaundecane	$NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$	12
Spermine	1,12-Diamino-4,9-diazadodecane	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	13
Thermospermine	1,12-Diamino-4,8-diazadodecane	$NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$	14
Homospermine	1,13-Diamino-4,9-diazatridecane	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_4NH_2$	15
Caldopentamine	1,15-Diamino-4,8,12-triazapentadecane	$NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$	16
Homocaldopentamine	1,16-Diamino-4,8,12-triazahexadecane	$NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$	17
Caldohexamine	1,19-Diamino-4,8,12,16-tetraazanonadecane	$NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$	18
Homocaldohexamine	1,20-Diamino-4,8,12,16-tetraazaeicosane	$NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$	18
	Tris(3-aminopropyl)amine	$[NH_2(CH_2)_3]_3N$	15
	Tetrakis(3-aminopropyl)ammonium	$[NH_2(CH_2)_3]_4N^+$	52

by comparing the physicochemical spectra of the isolated polyamines with those of chemically synthesized authentic amines.

Putrescine is always detected in this thermophile, though its amount is extremely small. Trace amounts of 1,3-diaminopropane were occasionally detected in the cell extracts. However, the presence of 1,3-diaminopropane as a precursor of norspermidine, thermine, and other longer polyamines was suggested by enzymatic studies since this compound served as a substrate for the aminopropyltransferase extracted from the thermophile.

Three triamines[11] and four tetraamines have been identified in the extracts as shown in Table 1. The major components in *T. thermophilus* grown at 75°C are two new tetraamines, thermine[12] and thermospermine.[14] They are an analogue and an isomer of spermine, respectively. Like 1,3-diaminopropane, some of the polyamines listed in Table 1 such as caldohexamine, putrescine, homospermine, and so on are present only in trace amounts. An example of polyamine composition is illustrated in Figure 1. The polyamine composition markedly varies depending on culture medium, culture temperature, growth stage, and other conditions.

Kneifel and Schoberth[13] reported the presence of a small amount of spermine in the thermophile cells. He detected spermine even in the cells grown in a synthetic medium, suggesting that this polyamine is also synthesized in *T. thermophilus* cells. A small amount of a homologue of spermine, homospermine,[15] was detected in the cell as shown in Figure 1. The presence of this minor polyamine was confirmed by measuring ^1H- and ^{13}C-NMR, IR, and mass spectra of this amine extracted from the thermophile and of the synthesized authentic compound as described later. The symmetrical isomer of this polyamine, 1,13-diamino-5,9-diazatridecane(=canavelmine), has been described in a bean.[19] However, this amine has not been detected in the cell-free extracts of the extreme thermophile.

T. thermophilus produces some polyamines longer than tetraamines. So far two straight-chained pentaamines and two hexaamines have been detected. Caldopentamine[16] is present in a considerable amount, and in the cells grown at extremely high temperatures such as 80°C or higher, this pentaamine was one of the major polyamines. The longest polyamine

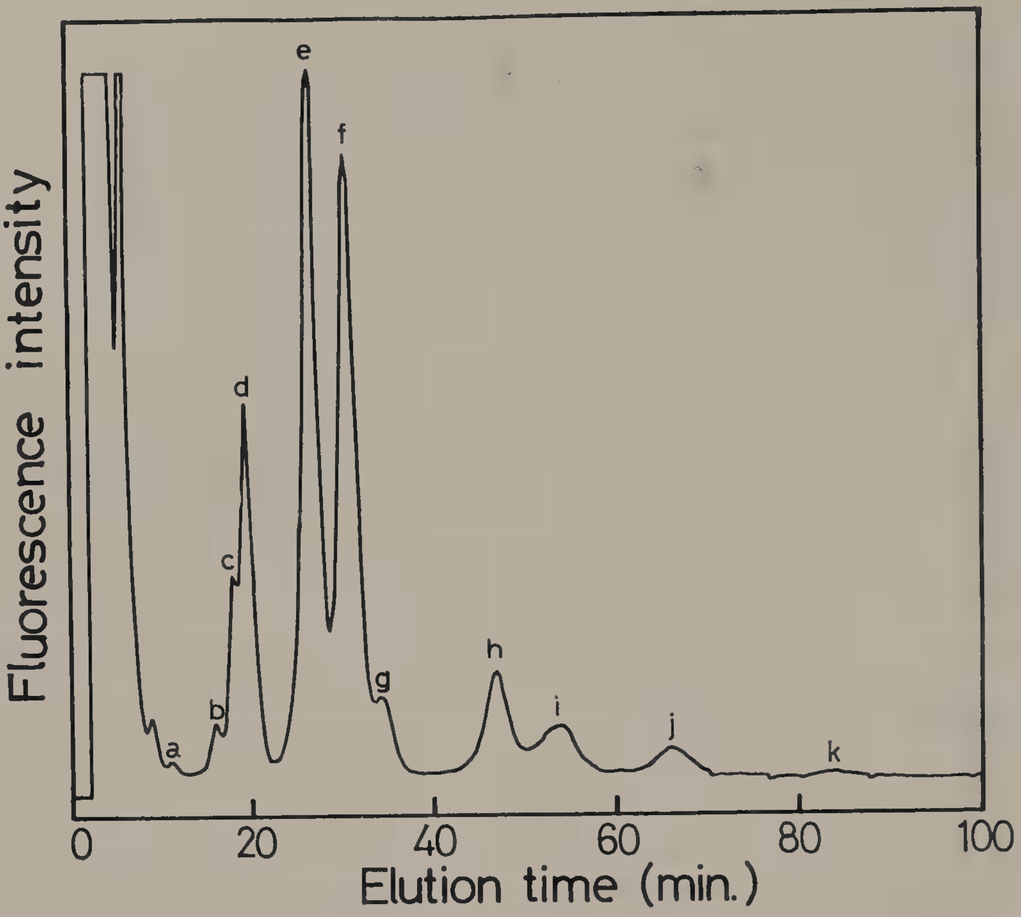


FIGURE 1. Polyamine composition of *T. thermophilus* grown at 75°C. a = putrescine, b = norspermidine, c = spermidine, d = *sym*-homospermidine, e = thermine (contaminated with tris[3-aminopropyl]amine), f = thermospermine (contamined with spermine), g = homospermine, h = caldopentamine, i = homocaldopentamine, j = tetrakis(3-aminopropyl)ammonium, and k = caldohexamine.

so far detected is homocaldohexamine.[17,18] No detectable amount of heptaamines was present in the cells.[18]

The most exciting finding in the studies of novel polyamines in *T. thermophilus* is the discovery of a quaternary ammonium, tetrakis(3-aminopropyl)ammonium in the extract[52] (for the chemical structure, see Table 1). The structure proposed, based on its NMR and mass spectra, was confirmed by chemical synthesis. A preliminary study for the biosynthesis of this quaternary ammonium suggested the presence of a corresponding tertiary amine as a precursor. The systematic name for the tertiary amine is tris(3-aminopropyl)amine. Unfortunately, the elution time of this tertiary amine is exactly the same to that of thermine on an analytical column chromatography used in our laboratory. However, this unique tetraamine containing a tertiary amino nitrogen was recently detected in an extract of *T. thermophilus* by Samejima et al.[53] using GC-MC. It is most likely that this tris amine is a biosynthetic precursor of the quaternary amine in the thermophilic cells.

Polyamine composition of other species of genus *Thermus* is similar to that of *T. thermophilus*. No polyamine longer than spermine was found in the extracts of moderately thermophilic bacteria, *B. stearothermophilus, B. acidocaldarius,* or *B. caldolyticus*. Tris(3-aminopropyl)amine and tetrakis(3-aminopropyl)ammonium were not present in these thermophilic *Bacilli*. An extreme, anaerobic thermophile, *C. thermohydrosulfuricum*, also produces thermine as a major component.[20] According to Kneifel and Schoberth,[13] who extensively studied the distribution of thermospermine in prokaryotes, these thermophilic eubacteria contain spermine, but no thermospermine. At present the thermophiles belonging to *Thermus* are the only organisms which produce thermospermine.

The novel polyamines found in *T. thermophilus* were also present in some mesophilic microorganisms and higher organisms. The presence of sym-homospermidine has been reported for mesophilic eubacteria,[21-23] cyanobacteria,[24] plants,[25-28] and animals.[29,30] Norspermidine and /or thermine are found in mesophilic bacteria,[31,32] algae,[33] euglena,[34,35] plant viruses,[36] sea animals,[37] shrimps,[37,38] and insects.[39]

III. POLYAMINES IN ARCHAEBACTERIA

Polyamine composition of archaebacteria is different from species to species. An extremely thermophilic and acidophilic archaebacterium, *S. acidocaldarius,* produces thermine as a major polyamine component.[15,40] The most abundant polyamine in *S. acidocaldarius* is spermidine. Norspermidine and small amounts of putrescine are also present in this bacterium. Spermine is also a major component in some strains of *S. acidocaldarius*; however, other strains contain only a trace amount of spermine which might be taken up from the yeast extract used in the culture medium, but not synthesized in the cell. For instance, 0.1 μmol of putrescine, 2.4 μmol of norspermidine, 13.6 μmol of spermidine, 4.9 μmol of thermine, and 0.1 μmol of spermine per gram of dry cell were present in the harvested cells of a strain of the acido-thermophilic archaebacterium, whereas 4.2 μmol of spermine per gram of dry cell was found in another strain.[15]

The polyamine composition of another acido-thermophilic archaebacterium, *Thermoplasma acidophilum*, is similar to that of mesophilic eubacteria; that is, only putrescine and spermidine are present in the cell.[15] Though many biochemical properties of these two acido-thermophilic archaebacteria are common to each other, their polyamine compositions are totally different.

The polyamine composition of other archaebacteria is also unique.[40] Homospermidine is the most abundant component in the cells of methane-producing archaebacteria.[41] A striking finding is that no polyamine is present in extremely halophilic archaebacteria.[23,42] The presence of caldopentamine has been reported for an alkalophilic, halophilic archaebacterium.[49]

IV. CHEMISTRY OF UNUSUAL POLYAMINES

Studies on new polyamines in *Thermus thermophilus* gave empirical rules for spectral assignments of polyamines.[39] Table 2 summarizes empirical assignments of ^{1}H- and ^{13}C-NMR spectra of polyamines.

The presence or absence of propyl group(s) can be judged by observing a signal at around 2.1 ppm in the proton-NMR spectrum. Likewise, the presence of a signal at around 1.8 ppm indicates the presence of butyl group(s) in the molecule. If both propyl and butyl groups are present in the molecule being studied, signals are found at 1.8 and 2.1 ppm in its ^{1}H-NMR spectrum, and at around 38 and 46 ppm in the ^{13}C-NMR spectrum. Intensities of these signals are roughly proportional to the number of amino group(s) and to twice the number of butyl group(s) in the polyamine molecule.

The presence of aminopropyl and aminobutyl ends can be confirmed by mass spectrometry. Since C–N bond is much stronger than C–C bond, fragments with mass numbers X-30 (CH_2NH_2), X-44 ($CH_2CH_2NH_2$), and X-58 ($CH_2CH_2CH_2NH_2$) (X is the molecular weight of the polyamine free base) can be seen along with the molecular peak at X in the mass spectrum if the polyamine contains aminobutyl terminal(s). If not, only the molecular and fragment peaks at mass number X, X-30, and X-44 (but not X-58) are predominant in the spectrum.

Polyamines containing tertiary amino nitrogen or quaternary ammonium nitrogen can be easily detected, since these compounds show an unique signal in ^{1}H-NMR (at around 3.5 to 3.6 ppm) and in ^{13}C-NMR (at around 55 ppm) in unusually low magnetic field.

Table 2
NMR SPECTRAL DATA FOR POLYAMINES

Methylene group	Chemical shift (ppm) ^{1}H-NMR	Chemical shift (ppm) ^{13}C-NMR
Propyl		
Adjacent to an amino nitrogen	3.2	37—39
Center in an aminopropyl terminal	2.1	23—24
Center between two aza nitrogens	2.1	24—25
Adjacent to an aza nitrogen	3.2	45—46
Butyl		
Adjacent to an amino nitrogen	3.2	37—39
Two next to an amino nitrogen	1.8	23—24
Two next to an aza nitrogen	1.8	22—23
Adjacent to an aza nitrogen	3.2	46—48
Adjacent to a tertiary amino nitrogen	3.5	52
Adjacent to a quaternary ammonium	3.6	57

Table 3
NMR SPECTRAL DATA FOR A POLYAMINE ISOLATED FROM *THERMUS THERMOPHILUS*

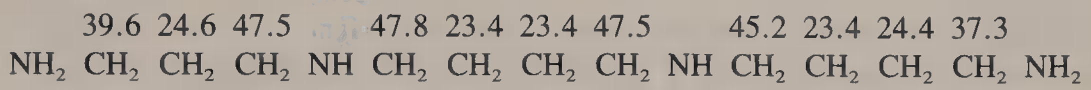
	Signal found	Relative intensity
^{1}H-NMR	1.7	4
	2.1	1
	3.1	6
^{13}C-NMR	23.4	3
	24.4	1
	24.6	1
	37.3	1
	39.6	1
	45.2	1
	47.5	3

One example of polyamine identification is as follows. Table 3 summarizes ^{1}H- and ^{13}C-NMR data of hydrochloride salt of a polyamine extracted and purified from *T. thermophilus*. Signals at 1.7 and 2.1 ppm in the proton-NMR indicate that both propyl and butyl groups are present in this polyamine, and the intensity ratio of these signals (4:1) indicates that the number of butyl groups in the molecule are twice that of propyl group(s). The intensity ratio of signals at 2.1 and 3.1 ppm also strongly supports this conclusion (a ratio of CH_2 adjacent to amino or aza nitrogen/propyl group is 6). The chemical formula should be

$$C_{11xn}N_{3xn+1}H_{25xn+3} \qquad (n = 1,2,3,....:)$$

Major signals found in mass spectrum of this polyamine were 216, 186 (= 216 − 30), 172 (216 − 44), and 158 (= 216 − 58). These data indicated that (1) the molecule is a tetraamine composed of one propyl and two butyl groups and (2) amino butyl terminal(s) is present in the molecule. Thus, homospermine or canavalmine could be proposed for the compound.

Finally, ^{13}C-NMR spectrum indicated that the chemical structure is asymmetrical, since no more than six signals should be found in the spectrum if the molecule is symmetric. Thus, the following structure can be deduced from these data. ^{13}C-NMR data strongly support the structure below and all ^{13}C-NMR signals found can be assigned as indicated.

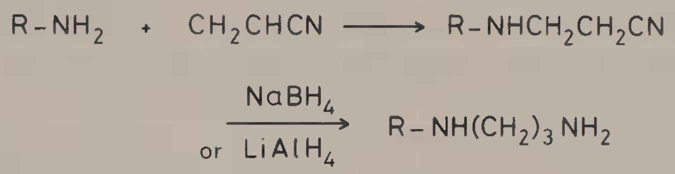


$$NH_2\ \overset{39.6}{CH_2}\ \overset{24.6}{CH_2}\ \overset{47.5}{CH_2}\ NH\ \overset{47.8}{CH_2}\ \overset{23.4}{CH_2}\ \overset{23.4}{CH_2}\ \overset{47.5}{CH_2}\ NH\ \overset{45.2}{CH_2}\ \overset{23.4}{CH_2}\ \overset{24.4}{CH_2}\ \overset{37.3}{CH_2}\ NH_2$$

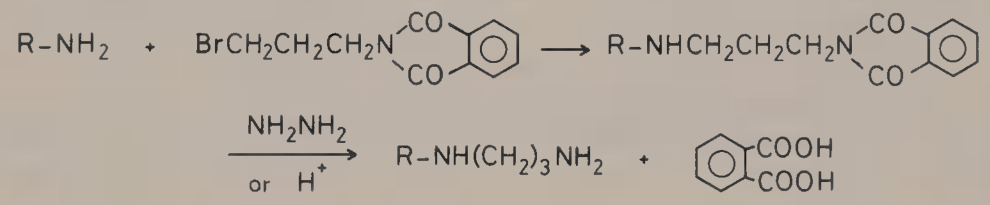
A trivial name homospermine was given to this compound and the structure was confirmed by chemical synthesis of the authentic tetraamine.[15]

Authentic polyamines can generally be synthesized by adding an aminopropyl or aminobutyl end group to an appropriate polyamine.[39] For the addition of an aminopropyl group, the following two reactions were used.

(1)



(2)



For the addition of an aminobutyl group, a reaction similar to that of *N*-(3-bromopropyl)phthalimide can be used.[39] Both *N*-(3-bromopropyl)phthalimide and *N*-(4-bromobutyl)-phthalimide are available from commercial reagent companies.

The reduction of CN group(s) can be made by using LiAlH$_4$ in an organic solvent. In our laboratory, sodium borohydride was also used to reduce CN group(s) in the presence of Co^{2+} ion in an aqueous solution.[39] Another sophisticated way for thermospermine synthesis has also been reported.[43]

If the yield is not concerned, "one pot synthesis" can be done without the isolation of intermediate compounds (for detail see Reference 39). The final product can be purified by a cation-exchange resin-column chromatography developed with the buffer used for analytical column chromatography. The resin used in our laboratory was CK-10S. Any ion-exchange resin used in automatic amino-acid analyzers can be used instead of CK-10S for the purification.

Tris(3-aminopropyl)amine was synthesized by reduction of 3,3′,3″-nitrilotris (propionamide) which is commercially available. This polyamine was also synthesized by mixing an excess amount of acrylonitrile with ammonium, followed by reduction. Tetrakis(3-aminopropyl)ammonium was synthesized by a reaction of NH$_2$-protected tris(3-aminopropyl)amine with *N*-(3-iodopropyl)phthalimide followed by deprotection with hydrazin. The details of the chemical synthesis of this quaternary ammonium is published elsewhere.[52]

The hydrochloric-acid salts of polyamines synthesized can be crystallized from a 50% hot ethanol solution, except for tris(3-aminopropyl)amine and tetrakis(3-aminopropyl) ammonium. These tertiary and quaternary amines are extremely hygroscopic, and no attempts to crystallize these amines have succeeded so far.

The unusual polyamines can be analyzed by HPLC or GC. In the author's laboratory, HPLC equipped with a CK-10S (or CK-10 U) column (4.9 mm diameter × 10 cm) has been used for the determination of polyamines.[39] The column is usually developed at 65°C with a K^+-citrate buffer, pH 5.0. The buffer is prepared by dissolving 32.4 g of hydrated potassium citrate, 65.0 mℓ of 1.000 N HCl, 150 g of KCl, 50 mℓ of 2-propanol, and 0.4 g of Brij 35 in H_2O, and the final volume is adjusted to 1000 mℓ. No pH adjustment was done. Polyamines eluted can be detected by reacting with ninhydrin or *o*-phthalaldehyde. Elution times for a variety of polyamines are illustrated in Figure 1.

Takeda et al.[44] determined the pK values of spermidine, thermine, spermine, and thermospermine by ^{15}N-NMR and ^{13}C-NMR titrations. The protonation sites of thermospermine were determined to be the order of N^{12}, N^1, N^8, and N^4, and their pK values were 11.6, 10.6, 9.4, and 8.3, respectively.

V. BIOCHEMISTRY OF POLYAMINES IN THERMOPHILES

Since it is known that polyamines stabilize nucleic acids and enzymes, it is most likely that polyamines in thermophiles play important roles in molecular mechanisms of thermophily. However, only little has been studied about the implications of polyamines in biochemical reactions at high temperatures.

The presence of a specific polyamine is essential for in vitro *protein synthesis* at high temperatures catalyzed by a cell-free extract of *T. thermophilus*.[45] The protein synthesis in vitro at 65°C was supported by 1 to 3 m*M* of spermine and thermospermine. No difference was observed between the reaction supported by spermine and that by thermospermine. Thermine also supported the in vitro protein synthesis, but the reaction was two thirds of that in the presence of spermine or thermospermine (Figure 2). Spermidine supported the cell-free protein synthesis only when a large amount of it (more than 10 m*M*) was added to the reaction mixture. Putrescine did not support the protein biosynthesis in vitro at all, and inhibited the activity of spermine, thermine, or thermospermine when added to the reaction mixture along with the tetraamine.

The detailed study revealed that the polyamines are important in stabilizing the ternary complex between aminoacyl-tRNA, messenger, and ribosomes. Exchange rate between free aminoacyl-tRNA and the bound one at high temperature was greatly reduced by the addition of a tetraamine.

Similarly, in vitro protein synthesis at high temperature catalyzed by the cell-free extract of an acido-thermophilic archaebacterium, *S. acidocaldarius*, requires the presence of thermine or spermine.[46,47] It can be speculated that protein biosynthesis in these extreme thermophiles is regulated by the intracellular concentrations of tetraamines.

Polyamine composition of thermophiles varies depending upon changes of environmental temperature. One example is shown in Figure 3. The amounts of polyamines composed of only propyl groups such as thermine, and caldopentamine in *T. thermophilus* increased with a rise of the incubation temperature. Also, longer polyamines were abundant in the thermophile incubated at higher temperature. Thus the most abundant polyamines in the cells grown at 80°C or higher were thermine and caldopentamine. In contrast, polyamines containing an amino butyl group, such as *sym*-homospermidine and thermospermine, were abundant in *T. thermophilus* cells grown at relatively low temperatures (such as 60°C).

In our preliminary experiments, aminopropyltransferase activity was found when a *T. thermophilus* extract was incubated with decarboxylated *S*-adenosylmethionine (= *S*-adenosyl(5′)-3-methylthiopropylamine) in the presence of a variety of polyamines found in this thermophile (the reaction product was not identified).

S. acidocaldarius extract did not accept thermine or spermine as a substrate of transaminopropylation in the presence of decarboxylated *S*-adenosylmethionine; this observation is in good agreement with the polyamine composition of the archaebacteria, since the corresponding pentaamine is not present in this microorganism.

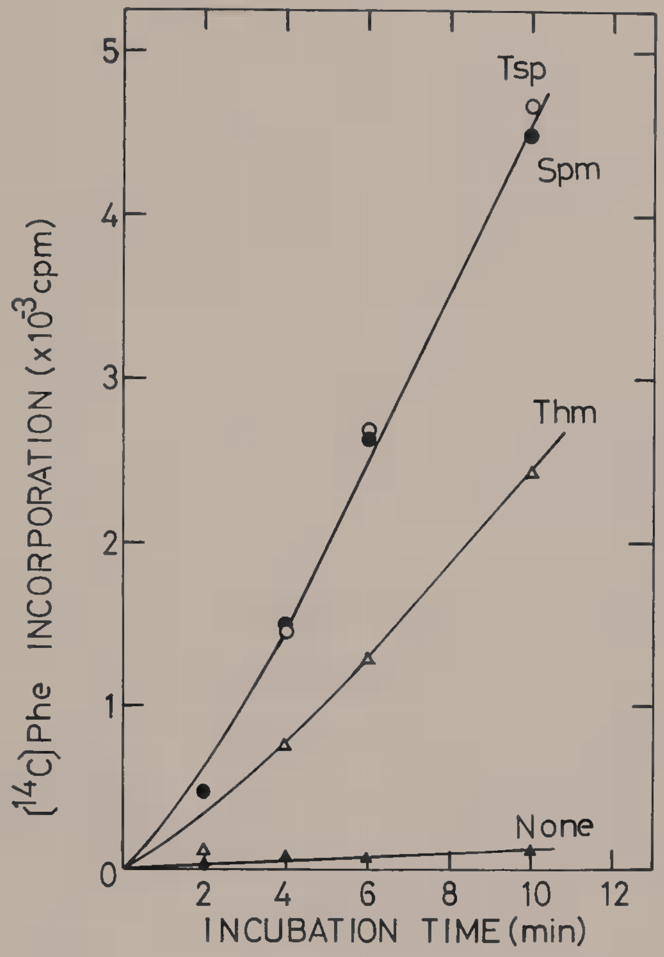


FIGURE 2. In vitro protein synthesis at 65°C. The reaction mixture contained the indicated polyamine (each 2 m*M*). (From Oshima, T., *Biochemistry of Thermophily,* Friedman, S. M., Ed., Academic Press, New York, 1978, 211. With permission.)

When radioactive putrescine was added to the cell-free extract of *T. thermophilus* in the presence of cold decarboxylated *S*-adenosylmethionine, labeled spermidine and thermospermine and/or spermine were found in the cells. Likewise the in vitro productions of norspermidine, thermine, and caldopentamine from 1,3-diaminopropane were observed. These findings suggest that polyamines are synthesized from the corresponding shorter polyamines by transaminopropylation in the thermophile cells.

When tris(3-aminopropyl)amine was added to the culture medium of *T. thermophilus,* tetrakis(3-aminopropyl)ammonium content in the grown cells slightly increased. Based on this observation, a hypothesis was proposed that the quaternary ammonium is synthesized from the tertiary amine.

Pösö and Paulin[48] extracted and partially purified ornithine decarboxylase from an anaerobic, extreme thermophile, *C. thermohydrosulfuricum,* and described its enzymatic properties. The thermostable enzyme was activated by the presence of GTP and was less sensitive to difluoromethylornithine, a potent inhibitor for eukaryote ornithine decarboxylase.

The polyamine composition of an acidothermophilic archaebacterium, *S. solfataricus,* closely resembles that of *S. acidocaldarius,*[49] that is, the major components are thermine and spermidine, and the minor components are norspermine and spermine. Recently, a report has been published on the purification and properties of a purified aminopropyltransferase from *S. solfataricus.*[50] The heat-stable enzyme is a trimer of identical 35 kdalton subunits. The enzyme specificity was in good agreement with the polyamine composition of this archaebacterium.

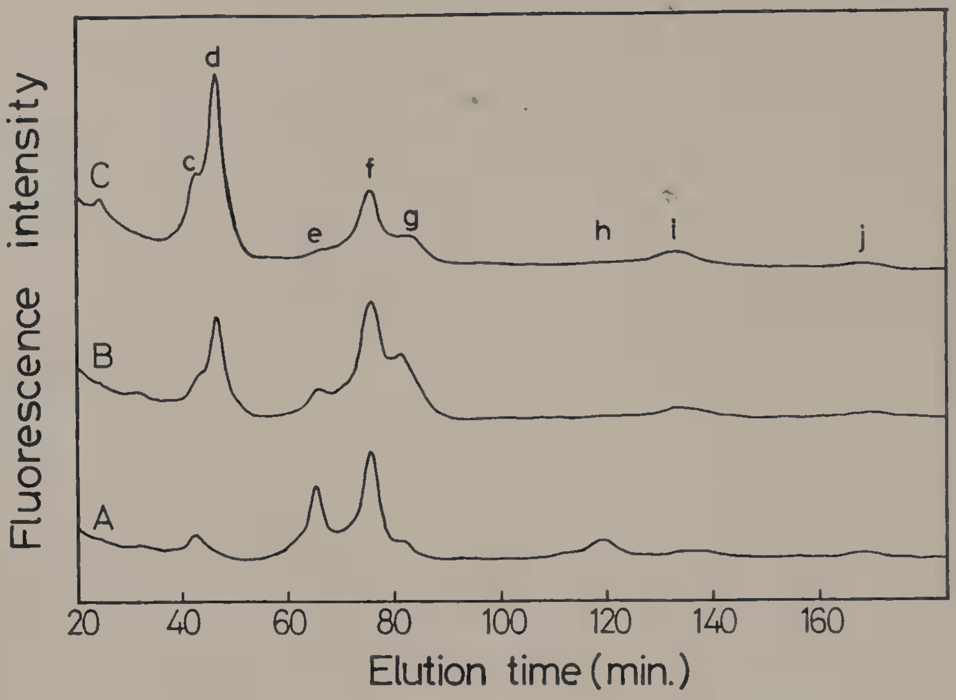


FIGURE 3. Changes of polyamine composition upon change of environmental temperature. The cells grown at 80°C in a synthetic medium were transferred into an incubator at 65°C. A = 0, B = 2, and C = 6 hr after the change of growth temperature; c = spermidine, d = *sym*-homospermidine, e = thermine (contaminated with tris[3-aminopropyl]amine), f = thermospermine (contaminated with spermine), g = homospermine, h = caldopentamine, i = homocaldopentamine, and j = tetrakis(3-aminopropyl)ammonium.

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Chapter 3

PROPYLAMINE TRANSFER REACTIONS IN THERMOPHILIC ARCHAEBACTERIA: ENZYMOLOGICAL ASPECTS AND COMPARATIVE BIOCHEMISTRY

Giovanna Cacciapuoti, Marina Porcelli, Gabriele Pontoni, and Vincenzo Zappia

TABLE OF CONTENTS

I. Introduction 48

II. Thermophilic Archaebacteria and Symmetrical Polyamines 48

III. Biogenetic Pathway of Polyamines in *Sulfolobus solfataricus* 51

IV. Assay Methods for Propylamine Transferase Reactions 51
  A. Separation of Polyamine Product from Polyamine Substrate 52
  B. Coupled Enzymatic Assays 52
  C. Separation of Polyamine Product from Decarboxy-AdoMet 52
  D. Separation of MTA from Decarboxy-AdoMet 52

V. Purification, Molecular Properties, and Substrate Specificity of Polyamine Transferase from *Sulfolobus solfataricus* 52

VI. Kinetics, Mechanism of the Reaction, and Inhibition Studies 55

VII. Conclusion and Perspectives 58

Acknowledgments 60

Addendum 60

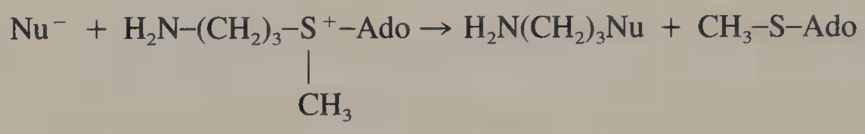
References 60

I. INTRODUCTION

Propylamine transferases represent a class of enzymes catalyzing the transfer of a propylamine group from *S*-adenosyl(5′)-3-methylthiopropylamine (decarboxy-AdoMet) to various amine acceptors,[1-4] according to the following equation:

S-Adenosyl(5′)-3-methylthiopropylamine + polyamine substrate →
5′-methylthioadenosine + polyamine product

The reaction is triggered by a nucleophilic attack on the C-3 methylene of the propylamine moiety adjacent to the sulfonium pole. The nucleophilic attack can be exerted either by an amino group of the substrate polyamine or, in the hypothesis of a double-displacement mechanism, by a nucleophilic group within the enzyme protein,[5] as illustrated below:



$$Nu^- + H_2N{-}(CH_2)_3{-}\underset{\displaystyle CH_3}{\underset{|}{S^+}}{-}Ado \rightarrow H_2N(CH_2)_3Nu + CH_3{-}S{-}Ado$$

In the latter case, a second nucleophilic attack on the propylaminated enzyme by the substrate polyamine is, in turn, responsible for the transfer of the propylamine fragment.

An alternative three-step pathway for the synthesis of spermidine, involving aspartic β-semialdehyde as propylamine donor, has also been described in some microorganisms and plants.[6]

Although numerous propylamine transferases with different features have been described in literature (Table 1), only few of them have been extensively purified and characterized. In 1958, Tabor et al.[7] provided the first evidence that decarboxy-AdoMet is the donor of aminopropyl groups in the biosynthesis of spermidine in cell-free extracts of *Escherichia coli*. Later on, this enzyme was purified to homogeneity by Bowman et al.[8]

Propylamine transferase activities have been reported in other bacteria, where these enzymes are also involved in the biosynthesis of unusual polyamine derivatives. The enzyme from *Pseudomonas acidovorans* preferentially reacts with the amino group distal to the hydroxyl function of 2-hydroxy-putrescine synthesizing 2-hydroxy-spermidine.[9] Furthermore, *Clostridium thermohydrosulfuricum*, a thermophilic eubacterium, is characterized by a peculiar propylamine transferase utilizing 1,3-diaminopropane as a substrate in the synthesis of sym-nor-spermine.[10]

In eukaryotes, the enzymatic synthesis of spermidine and spermine was first evidenced by Pegg and Williams-Ashman.[11] Soon afterward it was demonstrated that two distinct enzymes are operative and have been studied in several rat organs[4,12-16] and in human seminal plasma.[17] Both enzymes are also present in plants and yeast, such as *Vinca rosea*,[18] Chinese cabbage,[19] and *Saccharomyces cerevisiae*[20] (see Table 1).

The recent development of rapid and sensitive methods of polyamine analysis,[21-23] together with the utilization of affinity chromatography,[24,25] allowed the purification to homogeneity and a complete characterization of spermine and spermidine synthases from bovine brain[24] and rat ventral prostate,[25] respectively. More recently, a propylamine transferase with unusual molecular and kinetic properties has been purified to homogeneity in our laboratory from *Sulfolobus solfataricus*, an extreme thermophilic archaebacterium.[26]

II. THERMOPHILIC ARCHAEBACTERIA AND SYMMETRICAL POLYAMINES

S. solfataricus, previously named *Caldariella acidophila*,[27,28] is an extreme thermoaci-

Table 1
DISTRIBUTION IN NATURE OF AMINOPROPYL TRANSFERASES

Source	Enzyme	Ref.
	Prokaryotes	
Escherichia coli	Spermidine synthase	7,8
Micococcus denitrificans	Propylamine transferase	6
Pseudomonas acidovorans	Propylamine transferase	9
Clostridium thermohydrosulfuricum	Propylamine transferase	10
	Eukaryotes	
Rat ventral prostate	Spermidine and spermine synthases	4,11,25
Rat brain	Spermidine and spermine synthases	4,12
Rat liver	Spermidine and spermine synthases	4,14,15
Rat thymus	Spermidine and spermine synthases	4
Rat spleen	Spermidine and spermine synthases	4
Rat pancreas	Spermidine and spermine synthases	4
Rat kidney	Spermidine and spermine synthases	4
Human seminal plasma	Spermidine synthase	17
Bovine brain	Spermidine and spermine synthases	4,24
Saccharomyces cerevisiae	Spermidine and spermine synthases	20
Vinca rosea	Spermidine synthase	18
Chinese cabbage	Spermidine and spermine synthases	19
	Archaebacteria	
Sulfolobus solfataricus	Propylamine transferase	26

dophilic archaebacterium, isolated from an acidic hot spring in Agnano, Naples, optimally growing at 87°C. The morphology of this microorganism, investigated by electron microscopy, is shown in Figure 1a. The cells (0.5 to 1 μm of diameter) are generally spherical and contain a large number of dark granules, presumably ribosomes; a plasma membrane and an extracellular coat can be distinguished on the surface of the cell. Moreover, a loose irregular network of fibrils of DNA, evenly distributed throughout the cell wall, is observable. The fibrils, often branched and considerably variable in thickness, resemble the condensed DNA strands in the nucleoid of eubacteria. However, distinct nuclear areas could be identified only during cell division, that in *S. solfataricus* occurs through a mechanism of binary fission (Figure 1b).

Phylogenetic studies based on the composition of 16S ribosomal ribonucleic acid (RNA)[29,30] and on other biochemical features[30,31] indicate that *S. solfataricus* belongs to the primary kingdom of archaebacteria. The small size and structural simplicity, the absence of a nuclear membrane and organelles and their low DNA content, assimilate archaebacteria to prokaryotes.[30] On the other hand, archaebacteria are also related to the ancestor eukaryotes[32] in several properties, such as the presence of histone-like proteins,[33,34] the nature of the translation system,[35] the sequence of 5S RNA,[36] and that of ribosomal proteins.[37] On the basis of these properties archaebacteria can be truly envisioned as a third line of evolution completely distinct from eukaryotic and eubacterial lines.[38] They include three major phenotypes confined to few ecological niches, such as thermal habitat for extreme thermophiles, saturated brine for extreme halophiles, stagnant water, rumen of animals, and hydrothermal environments for methanogens.[30]

Among thermophiles, *S. solfataricus*, has a remarkably high growth temperature,[27] Moreover, the polyamine pool of this microorganism is characterized by two unusual polyamines,

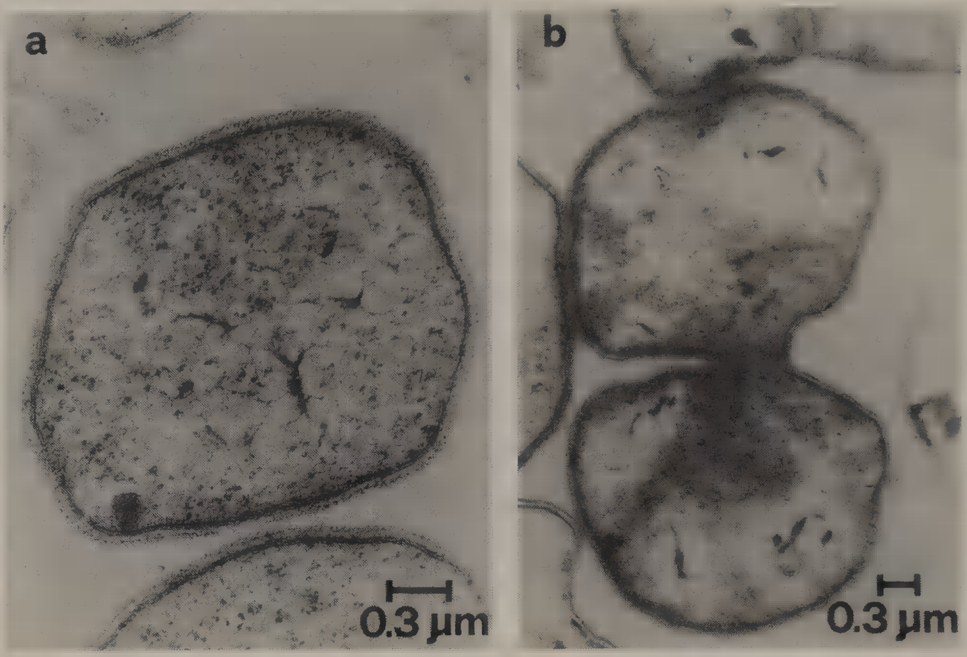


FIGURE 1. (a) *Sulfolobus solfataricus* cell osmium-fixed. Unit membrane and extracellular structure resolved. (b) Replication by binary fission (osmium-fixed).

Table 2
POLYAMINES PATTERN IN *SULFOLOBUS SOLFATARICUS*

Polyamines	Cellular concentration (µmol/g wet cells)
Putrescine	0.074
Sym-nor-spermidine	1.8
Spermidine	8.2
Sym-nor-spermine	15.6
Spermine	0.02

sym-nor-spermidine and sym-nor-spermine.[39] Oshima first reported the presence of sym-nor-spermine in several extreme thermophilic eubacteria as *Thermus thermophilus, T. flavus*, and *T. aquaticus*[40] (for further details on the subject, see Chapter 2), while the occurrence of sym-nor-spermidine was demonstrated in *S. solfataricus* by our group.[39] It is interesting to note, in this respect, that in bacteria the polyamine pattern is apparently related to their growth temperature.[41] Indeed, sym-nor-spermine represents the major component of polyamine pattern in thermophiles as indicated in Table 2.[41]

The high content of polyamines in *S. solfataricus* and the observation that tetraamines are required for the stimulation, in vivo, of protein synthesis in the extreme thermophile *T. thermophilus*[42] suggest that these molecules play a role in the thermophilicity and thermostability. The peculiar carbon-chain length and charge distribution of symmetrical polyamines could facilitate specific interactions with nucleic acids, resulting in a protection of DNA against thermal denaturation.[43] The observation that sym-nor-spermine increases the melting temperature of DNA from *Aerobacter aerogenes* and from calf thymus[44] supports this hypothesis. On the other hand, the G + C content of DNA from *S. solfataricus* is only 42%[27] and does not account for its stability at the optimal growth temperature of the microorganism (87°C). In this respect, the existence in vivo of a specific stabilizing effect of

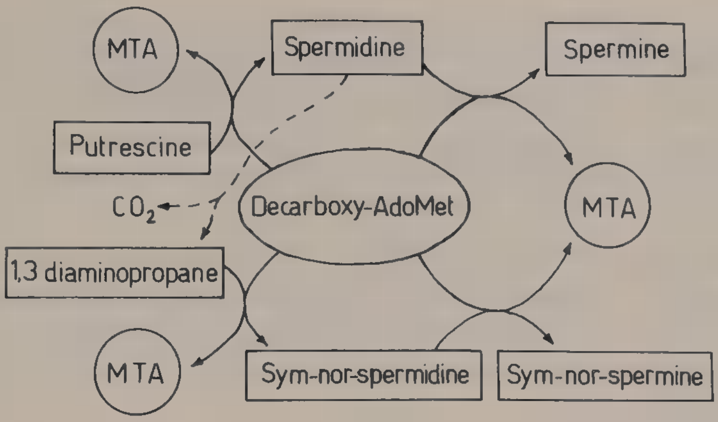


FIGURE 2. Propylamine transfer reactions in thermophiles.

sym-nor-spermine on the homologous DNA, according to the model proposed by Liquori et al.,[45] is a matter of investigation.

III. BIOGENETIC PATHWAY OF POLYAMINES IN *SULFOLOBUS SOLFATARICUS*

The biosynthesis of sym-nor-spermidine and sym-nor-spermine has been investigated in *S. solfataricus*.[43] When the cells were grown in the presence of putrescine and spermidine, labeled in the tetramethylene moiety, the radioactivity was recovered only as spermidine, while when the cells were supplied by methionine or spermidine labeled in the propylamine moiety, the radioactivity was recovered in sym-nor-spermidine and sym-nor-spermine. On the basis of such experiments, the biosynthetic pathway reported in Figure 2 was proposed.

In thermophiles the biosynthesis of spermidine closely resembles the pathway occurring in mesophilic microorganisms[1] and mammalian tissues,[3,4] in that putrescine supplies the 1,4-diaminobutane moiety and methionine donates the propylamine group. On the contrary, for the biosynthesis of sym-nor-spermidine and sym-nor-spermine an unusual reaction is required in which spermidine plays the role of precursor of the trimethylene moiety. In fact 1,3-diaminopropane, probably originated from spermidine breakdown, is rapidly propylaminated to form sym-nor-spermidine, the latter being in turn converted into sym-nor-spermine by an analogous reaction. The role of 1,3-diaminopropane as a free intermediate in this chain of reactions has been hypothesized, even though appreciable amounts of this diamine were not detectable in cellular extracts. 1,3-Diamino-propane represents indeed a catabolic product of spermidine oxidation in several bacterial species.[46]

The main feature of this pathway is that the entire molecular skeleton of the symmetrical polyamines derives from decarboxy-AdoMet in that the propylamine moiety coming from spermidine in turn also originates from the sulfonium compound.

IV. ASSAY METHODS FOR PROPYLAMINE TRANSFERASE REACTIONS

The laborious and time-consuming assays for propylamine transfer reactions, together with the lack of commercially available decarboxy-AdoMet, hampered rapid developments in this field in the past. Only recent procedures, which allow a more precise separation and quantitation of the products and/or substrates of the reaction, allowed more sophisticated approaches.

Based on the overall reaction, the transfer of the propylamine group can be monitored by several assay procedures due to the following strategies.

A. Separation of Polyamine Product from Polyamine Substrate

The first developed assay procedure implies separation of labeled spermidine, spermine, and putrescine by means of several techniques such as paper chromatography,[8] thin-layer chromatography,[8] ion-exchange chromatography,[47] and paper electrophoresis.[8] Spermidine was also selectively quantified fluorometrically.[8] All these procedures are limited by the poor resolution and the low accuracy due to the uneasy handling.

More rapid and simplified procedures have been developed more recently by means of high performance liquid chromatography (HPLC) for the separation of either radioactive polyamines[2] or benzoyl-polyamines.[48] The latter is also the only adequate procedure for the determination of sym-nor-spermidine and sym-nor-spermine in that their labeled standards are not commercially available.

All these methods are generally inadequate for the assay of the enzyme from thermophiles since its high Km polyamine/Km decarboxy-AdoMet ratio results in a very low yield of the polyamine product which, in turn, lowers the sensitivity under the limits required for the detection of the product.

B. Coupled Enzymatic Assays

V5′-Methyethioddenosine (MTA) can be determined spectrophotometrically by means of adenosine deaminase from *Aspergillus oryzae*,[49] which selectively recognizes the thioether as substrate while the sulfonium compound is not cleaved. Alternatively, spermidine can be measured by its oxidation with spermidine dehydrogenase from *Serratia marcescens*.[50,51] Interferences by MTA- and/or spermidine-metabolizing enzymes, respectively, limit these methods to rather purified preparations. Moreover, both these procedures are not suitable for enzymes from thermophilic organisms because of the thermolability of ancillar enzymes under the high assay temperature.

C. Separation of Polyamine Product from Decarboxy-AdoMet

In the procedure proposed by Raina et al.,[52] the radioactive polyamine product is separated from decarboxy-AdoMet, labeled in the propylamine moiety, by using discs of weak cation-exchange paper. The method is quite rapid and reproducible, the only disadvantage being the lack of commercially available properly labeled decarboxy-AdoMet.

D. Separation of MTA from Decarboxy-AdoMet

The significant charge difference between the two nucleosides permits easy separation. A method proposed by Raina et al.,[23] as a development of a previous procedure by Hibasami and Pegg,[21] implies the use of a weak cation-exchange resin. This assay can also be usefully utilized with crude extracts, since, although MTA may undergo further catabolism, its degradation products are not retained under the chromatographic conditions employed. All polyamines can be tested as substrate by this method, since MTA is a common product of all propylamine-transfer reactions. This feature makes the method suitable for the thermophilic enzyme. However, the HPLC technique, associated with the derivatization of polyamines with benzoyl chloride[48] should be employed for a precise identification of the polyamine product.

V. PURIFICATION, MOLECULAR PROPERTIES, AND SUBSTRATE SPECIFICITY OF PROPYLAMINE TRANSFERASE FROM *SULFOLOBUS SOLFATARICUS*

The enzyme from *S. solfataricus* has been purified to homogeneity by affinity chromatography on decarboxy-AdoMet-Sepharose and phenyl Sepharose as major purification steps.[26] The purification procedure is reported in Table 3. The recovery of the enzyme activity was

Table 3
PURIFICATION PROCEDURE OF PROPYLAMINE TRANSFERASE FROM *SULFOLOBUS SOLFATARICUS*

Step	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	19,400	0.0032	1	100
55—75% Ammonium sulfate fractionation	4,200	0.010	3.1	70
DEAE-cellulose	1,386	0.024	7.5	55.3
Sephacryl S-200	228	0.121	37.89	44.5
Decarboxy-AdoMet-Sepharose	38.5	0.449	140.3	27.9
Phenyl-Sepharose	6.08	2.059	643	20.17

Note: A unit is the amount of the enzyme which forms 1 μmol of MTA/min at 78°C.

Table 4
MOLECULAR PROPERTIES OF PROPYLAMINE TRANSFERASE FROM *SULFOLOBUS SOLFATARICUS*

Molecular weight	110,000
Quaternary structure	Trimeric
Isoelectric point	5.3
Optimum pH	7.5
Optimum temperature	90°C
Specific activity	2.05 μmo/min/mg

20% and the final preparation had a specific activity (2.05 μmol of MTA formed per minute per milligram of protein) about 640-fold higher than that of the crude supernatant (Table 3).

The activity ratio towards the polyamine acceptors (1,3-diaminopropane, putrescine, and sym-nor-spermidine) are constant throughout the purification steps, thus indicating that a single enzyme is responsible for the sequence of the three propylamine transfer reactions.

The enzyme is cytosolic and no activity is associated with the cellular particulate. Furthermore, propylamine transferase was not induced by the addition of polyamines to the standard culture medium.

Several physical and kinetic properties of the homogeneous enzyme are given in Table 4. It has a molecular weight of 110,000 daltons as indicated in Figure 3 and is composed of three apparently identical subunits of 35,000 daltons. It is worth noting that both prokaryotic and mammalian propylamine transferases are dimeric proteins.[4,8] This difference in quaternary structure is probably related to the phylogenesis of archaebacteria that are genetically distinct from both prokaryotes and eukaryotes.[38]

Thiol groups are not involved in the catalytic process since the enzyme, like *Escherichia coli* spermidine synthase,[8] is not affected by alkylating, mercaptide-forming, or oxidizing thiol reagents. Moreover, the isoelectric point and optimum pH are similar to the corresponding enzymes from eukaryotes.

As observed in several enzymes from thermophilic microorganisms,[53] the optimum temperature of propylamine transferase is quite high (90°C), similar to the optimal growth temperature of the bacterium (87°C) (Figure 4). The enzyme also shows a remarkable thermostability. No loss of activity is observable after preincubation for 1 hr at 100°C. Such property is uncommon even when compared to other enzymes from thermophiles;[54] the enzyme indeed appears stable both at low protein concentration and at the highest level of

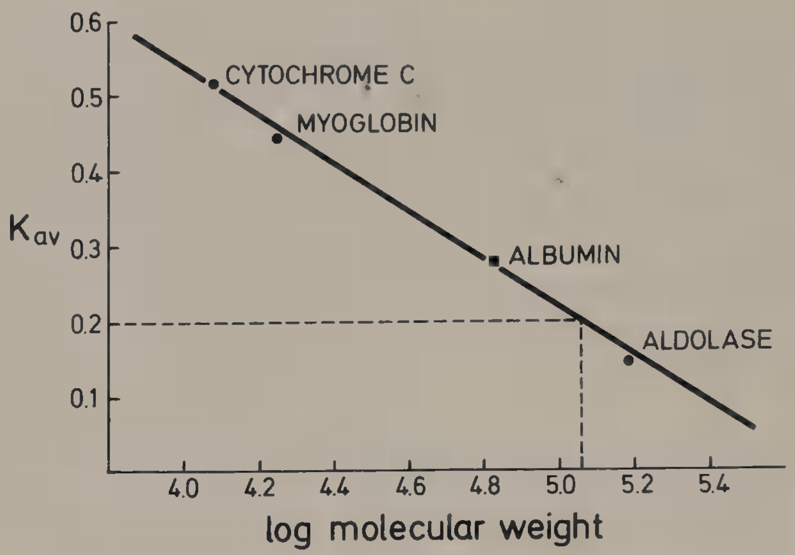


FIGURE 3. Molecular weight of propylamine transferase from *Sulfolobus solfataricus*.

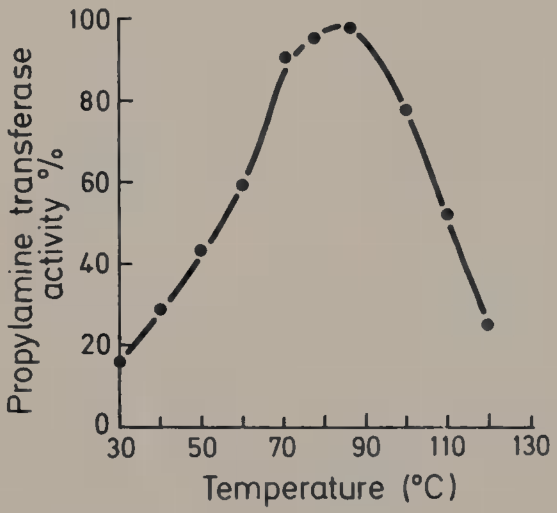


FIGURE 4. Effect of temperature on propylamine transferase activity. The assay mixture was prepared as described in Reference 66. The incubation was carried out for 3 min at the indicated temperature in air-tight sealed tubes.[66]

purity. No protection against thermal inactivation at 120°C is observable by incubating the enzyme in the presence of polyamines.

The specificity of the enzyme is significantly different when compared with other propylamine transferases[8,55] in that it is able to transfer the propylamine group from decarboxy-AdoMet to a primary amino group linked to a trimethylenic as well as to a tetramethylenic chain. This observation is in agreement with the incorporation of 1,3-diaminopropane into sym-nor-spermine observed in vivo in *C. thermohydrosulfuricum*[10] and confirms the unusual biogenetic pathway previously proposed.[43]

Table 5 reports the enzyme activity in the presence of potential propylamine acceptors. Among the substrates assayed, 1,3-diaminopropane appears to be the most active, followed by putrescine, sym-nor-spermidine, and spermidine. 1,5-Diaminopentane and sym-nor-spermine are very poor substrates, while all the other compounds tested are virtually inactive.

Table 5
SUBSTRATE SPECIFICITY OF PROPYLAMINE TRANSFERASE FROM *SULFOLOBUS SOLFATARICUS*

Substrates	Enzyme activity (nmol MTA formed/min/mg)	Relative activity (%)
1,3-Diaminopropane	1800	100
Putrescine	662	36.7
1,5-Diaminopentane	10.8	0.6
1,9-Diaminononane	nd	<0.1
1,10-Diaminodecane	nd	<0.1
Sym-nor-spermidine	316	17.5
Spermidine	124	6.8
Sym-homospermidine	nd	<0.1
Sym-nor-spermine	50	2.7
Spermine	nd	<0.1
Caldopentamine	nd	<0.1
Homocaldopentamine	nd	<0.1

Note: nd = not detectable.

Table 6
KINETIC PARAMETERS OF PROPYLAMINE TRANSFERASE FROM *SULFOLOBUS SOLFATARICUS*

	Kinetic constants	
Polyamines	Km (m*M*)	Vmax (nmol MTA formed/min/mg)
1,3-Diaminopropane	1.675	2050
Putrescine	3.85	662
Sym-nor-spermidine	0.95	316
Spermidine	1.54	124

On the basis of a kinetic analysis, the Km values of the most active substrates have also been calculated (Table 6).

The affinity of the enzyme for decarboxy-AdoMet (Km = 7.9 μ*M*) is similar to that of rat prostate (Km = 1.1 μ*M*) and *E. coli* (Km = 2.2 μ*M*) spermidine synthase,[8,25] but significantly lower when compared to bovine brain spermidine synthase (Km = 0.3 μ*M*)[56] and spermine synthase (Km = 0.6 μ*M*).[24] The Km value, when compared with the cellular amounts of decarboxy-AdoMet in *S. solfataricus*,[26] indicates that the sulfonium compound is not saturating in vivo, thus suggesting a regulatory role of this metabolite on the reaction rate. However, for the more precise evaluation, the estimation of the cellular content of 1,3-diaminopropane should be performed.

VI. KINETICS, MECHANISM OF THE REACTION, AND INHIBITION STUDIES

In recent years many efforts have been directed to elucidate the mechanism(s) of propylamine transfer reactions. The overall reaction can be envisaged as a nucleophilic attack of a polyamine amino group to a sulfonium pole-adjacent methylene (Figure 5). The latter is indeed a good electrophile, being electron deprived by the positively charged sulfur. Nevertheless, examining the mechanism of enzyme action, a direct polyamine attack on decarboxy-AdoMet must be considered only in the hypothesis of a sequential mechanism,

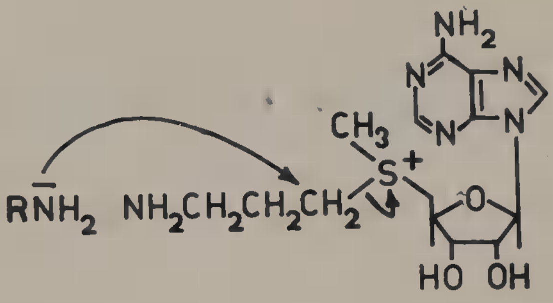


FIGURE 5. Nucleophilic attack of polyamine on decarboxy-AdoMet in the hypothesis of a sequential mechanism.

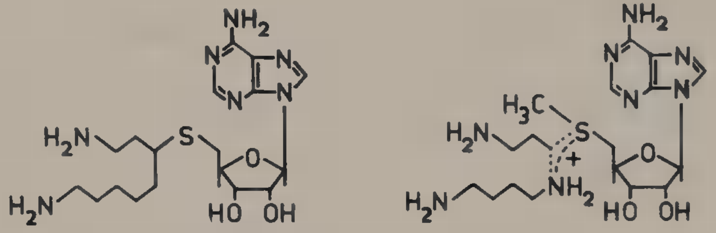


FIGURE 6. Molecular structure of AdoDATO in comparison with a propylamine transferase transition-state in the hypothesis of a sequential mechanism.

irrespective of whether it is random or ordered.[57] On the contrary, if a ping-pong mechanism is operative, two subsequent nucleophilic attacks must be taken into account; the first is due to a nucleophilic amino-acidic residue or coenzyme within the enzyme protein and results in the formation of a propylamine-enzyme covalent intermediate, which in turn undergoes a second attack exerted by the polyamine. In both cases MTA serves as a leaving group.[58] The selection of the C-3 methylene, instead of the two other chemically equivalent sulfur-adjacent carbons, is an effect of a specific enzymatic interaction. In fact the electron withdrawal is uniformly distributed in the three directions from the sulfonium pole.[59]

E. coli spermidine synthase is by far the most studied propylamine transferase as far as the enzymatic mechanism is concerned, although the results obtained are not completely univocal. Coward et al.[60] evidenced a substrate excess inhibition of decarboxy-AdoMet, by means of a kinetic approach. Later on, Billington et al.[61] ruled out any intramolecular rearrangement step during the enzymatic process by means of an isotope labeling technique. A more complete kinetic approach[2,58] has been performed in our laboratory, showing parallel patterns in the double reciprocal plots, obtained by varying the concentration of each substrate while keeping the other at several fixed levels. This result leads to the conclusion that the mechanism is of a double-displacement type. Moreover, decarboxy-AdoMet was demonstrated to act as a dead-end inhibitor by combining with the propylamine-enzyme complex.[58] Conversely, a sequential mechanism was proposed by Golding and Nassereddin,[62] based on rather complex NMR experiments performed in vivo. A sequential mechanism was also proposed on the basis of the potent inhibitory action of *S*-adenosyl(5′)-3-diaminothiooctane (AdoDATO, see the structure reported in Figure 6),[63] which was supposed to act as a transition-state analogue (TSA) inhibitor.[64] However, by means of more sophisticated kinetic experiments, the powerful inhibition exerted by AdoDATO was found more consistent with an inhibited ping-pong mechanism rather than a TSA inhibition.[65]

Spermidine synthase from bovine brain was convincingly proposed as a sequential enzyme based on a complete kinetic characterization carried out on the homogeneous protein,[56]

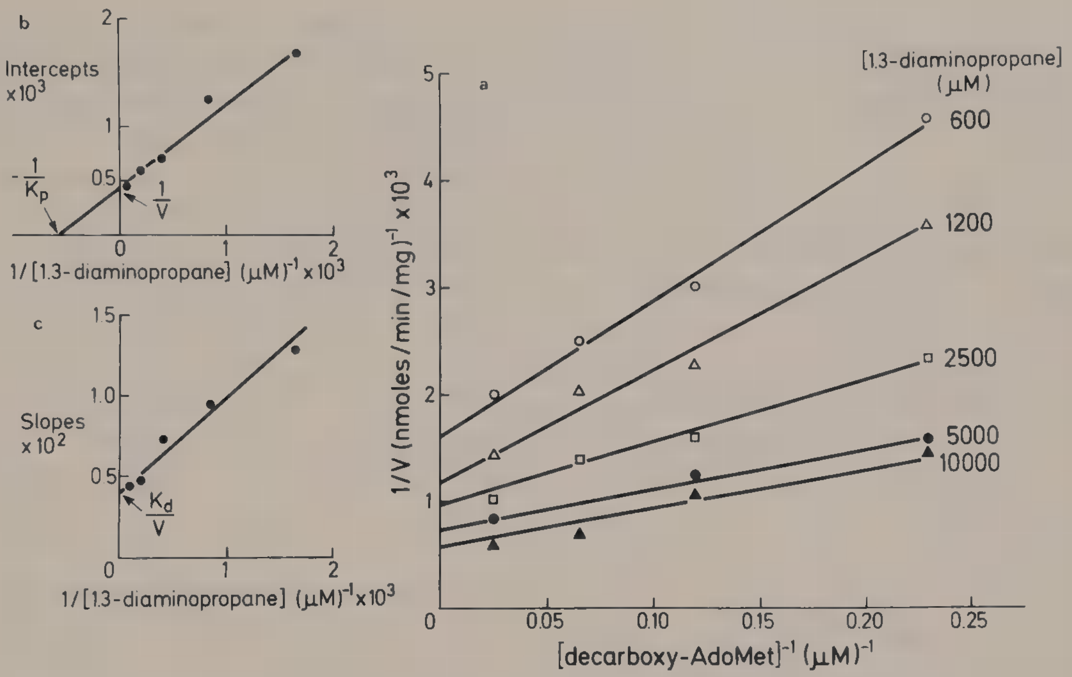


FIGURE 7. Plot of the reciprocal of the initial velocity (V) vs. the reciprocal of the μ*M* concentration of decarboxy-AdoMet at the various indicated fixed levels of 1,3-diaminopropane.

although it is not yet clear whether a random or ordered process takes place. Kinetic experiments have also been performed on homogeneous spermine synthase from the same source and a sequential mechanism was again proposed.[24] This enzyme was not affected by decarboxy-AdoMet excess inhibition, unlike all spermidine synthases so far known from eukaryotic[60] and prokaryotic sources.[58]

The results of kinetic experiments with homogeneous propylamine transferase from *S. solfataricus* are indicative for a sequential mechanism.[66] Initial velocity studies were indeed carried out at various concentrations of decarboxy-AdoMet in the presence of fixed concentrations of 1,3-diaminopropane. As shown in Figure 7, a linear intersecting pattern was obtained. No decarboxy-AdoMet excess inhibition was observable in this enzyme, which, in this respect, resembles spermine more than spermidine synthases.

Although this topic deserves further investigation, in our opinion it is entirely possible that two different mechanisms are operative in bacterial and in eukaryotic propylamine transferases. In this respect the behavior of the enzyme from *S. solfataricus* is of relevance in that it acts through a sequential mechanism like the eukaryotic counterpart.

Several studies concerning enzyme-substrate interactions have been carried out using analogues of decarboxy-AdoMet as inhibitors of propylamine transferases from both eukaryotic and prokaryotic sources.[67-70] In eukaryotes the same decarboxy-AdoMet binding sites have been proposed for spermidine and spermine synthase. Both enzymes appear to require the amino group in the 6-position of the adenine ring, while nitrogens 3 and 7 are not relevant. Moreover, modification of the C-1 position of the propylamine fragment of the sulfonium compound by addition of a carboxyl group or by removal of the amino group is compatible with recognition. Finally, a sulfonium center is of importance for both enzymes, although its substitution with a sulfoxide or sulfone group is compatible with recognition.[67]

MTA exerts a powerful competitive inhibition on the reaction and studies with adenine-modified analogues indicate recognition sites similar to that identified for decarboxy-AdoMet. Conversely, replacement of the methyl group with ethyl, propyl, or isobutyl does not significantly affect inhibitory activity.[67]

Similar recognition sites for decarboxy-AdoMet have also been proposed for *E. coli* propylamine transferase[58] with few differences concerning the C-1 position of propylamine fragment where an amino group is required and the addition of a carboxyl group is not tolerated. Propylamine transferase from *S. solfataricus* differs from both prokaryotic and eukaryotic homologues in terms of recognition (see Table 7). The requirement for an amino group in C-1 position assimilates this enzyme to the prokaryotic counterpart. Conversely, the powerful inhibition exerted by *S*-adenosyl-3-thiopropylamine suggests that the sulfonium pole is not involved in the binding, unlike both the prokaryotic and eukaryotic enzymes.[66]

Many MTA analogues have been tested as inhibitors of the thermophilic enzyme. The inhibition exerted by 5′-methylthiotubercidin indicates that the N-7 of the purine ring is not relevant in the recognition process. Furthermore, the substitution of the imidazole of the purine base by a pyrazole ring as in 5′-methylthioformycin, results in a complete loss of inhibitory activity. Indeed the protonation of N-7, allowed only in the pyrazole ring at physiological pH, may prevent the binding with the enzyme. On the other hand, the absence of inhibitory effect by 5′-methylthioinosine, as well as the drop of inhibition by N-6 substituted derivatives, confirms the relevance of the purine amino group in the binding with the enzyme.[66]

In the light of a comparative enzymological analysis, particular attention must be paid to dicyclohexylamine, a well-known potent and specific inhibitor of *E. coli*[71] and rat prostate[72] spermidine synthases. This compound inhibits only slightly propylamine transferase from *S. solfataricus*. The different behavior of the thermophilic enzyme with respect to specific inhibitors further confirms the taxonomic position of archaebacteria.

Finally, a screening of several diamines as inhibitors (Table 7) indicated that the length of the aliphatic spacer between the primary amino groups is critical, in analogy with eukaryotic and prokaryotic enzymes.[66]

VII. CONCLUSION AND PERSPECTIVES

The peculiar substrate specificity of the purified propylamine transferase from *S. solfataricus*, confirms the pattern proposed in Figure 2, although the elucidation of the metabolic origin of 1,3-diaminopropane from spermidine deserves further investigation.

On the basis of the inhibition studies carried out with several analogues of polyamines and decarboxy-AdoMet, at least four binding sites have been proposed between the two substrates and the enzyme protein (see Figure 8). The sulfonium compound is identified by means of the two amino groups in the purine ring and in the propylamine moiety, while the recognition of the polyamine involves the two correctly spaced amino groups.

The sequential mechanism proposed on the basis of initial velocity studies assimilates propylamine transferase from *S. solfataricus* to eukaryotic spermine synthase more than to its prokaryotic homologue. In this respect, a straightforward method has been published based on the study of the stereochemical modifications at the C-3 methylene of the propylamine fragment during the reaction.[73] This procedure will probably give a final word on the mechanism(s) of propylamine transfer reactions.

An interesting perspective suitable to confirm the unique taxonomy of archaebacteria is the investigation of the degree of sequence homology among propylamine transferases from different sources. A complete elucidation of the primary structure of *S. solfataricus* propylamine transferase as well as of its mesophilic counterparts will also shed new light on the problem of thermostable proteins. In this respect, a small increase in hydrogen bonding or in salt bridging within the thermophilic enzyme proteins, when compared with mesophilic homologues, has been proposed as a sufficient explanation for enhanced thermostability.[74] In this instance, though, the differences in the quaternary structure of propylamine transferase may also play a significant role.

Calculation and comparison of parameters of activation thermodynamics such as ΔG^*, ΔS^*, and ΔH^* among propylamine transferases from thermophilic and mesophilic organisms

Table 7
INHIBITION OF PROPYLAMINE TRANSFERASE BY SUBSTRATE AND PRODUCT ANALOGUES

Compound	Conc (m*M*)	Inhibition (%)
Analogues of decarboxy-AdoMet		
S-Adenosyl(5′)-3-methylthiopropanol	0.5	0
S-Inosyl(5′)-3-methylthiopropanol		
S-Adenosyl-3-thio-1,8-diaminooctane	0.5	0
	0.021	48
Analogues of polyamines		
1,3-Diaminopropan-2-ol	1.0	0
Dicyclohexylamine	1.0	27
1,8-Diaminooctane	1.0	0
1,9-Diaminononane	1.0	0
1,10-Diaminodecane	1.0	0
Analogues of MTA		
MTA	0.1	100
n-Propylthioadenosine	0.1	45
5′-Isobutylthioadenosine	0.1	35
5′-Ethylthioadenosine	0.1	40
S-Adenosylhomocysteine	1.0	0
S-Adenosyl-3-thiopropylamine	0.1	100
Sinefungin	0.1	58
A9154C	0.1	43
5′-Dimethylthioadenosine	0.5	0
N^6-Methyl-MTA	0.1	44
N^6-Dimethyl-MTA	0.1	23
N^6-Allyl-MTA	0.1	38
N^6-Isopropyl-MTA	0.1	23
5′-Methylthioinosine	0.5	0
5′-Methylthiotubercidin	0.1	100
5′-Methylthioformycin	0.5	0
5′-Chloroformycin	0.5	0

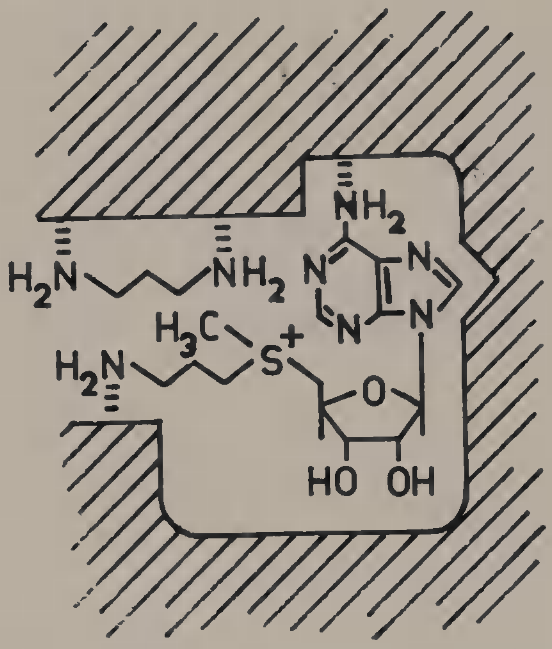


FIGURE 8. Proposed enzyme-substrates binding sites.

will also be useful tools for a better understanding of the protein-substrate interaction in thermophilic enzymes.[75] The topic is of interest in that a peculiar energetics of this process can be expected, due to the extremely high temperature required for the reaction.

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ADDENDUM

Since submission of this chapter a paper has been published by Orr G. R. et al.[76] on the mechanism of aminopropyl transfer reactions. On the basis of stereochemical studies it has been proposed that *E. coli* spermidine synthase reaction proceeds *via* a single-displacement mechanism similar to that reported for spermidine and spermine synthase from mammalian tissues.

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Chapter 4

MICROBIAL MUTANTS DEFICIENT IN POLYAMINE SYNTHESIS

Celia White Tabor and Herbert Tabor

TABLE OF CONTENTS

I. *Escherichia coli* 64
  - A. *speA* (Arginine Decarboxylase) and *speB* (Agmatine Ureohydrolase) 64
  - B. *speC* (Ornithine Decarboxylase) 64
  - C. *speE speD* Operon (Spermidine Synthase and Adenosylmethionine Decarboxylase) 64
  - D. *cadA* (Lysine Decarboxylase) 65
  - E. *metK* (Adenosylmethionine Synthetase) 65
  - F. *pat* (Putrescine Aminotransferase) 65
  - G. Other *Escherichia coli* Enzymes 65
  - H. Characteristics of Polyamine-Deficient *Escherichia coli* 65

II. *Saccharomyces cerevisiae* 66
  - A. *spe1* (Ornithine Decarboxylase) 68
  - B. *spe2* (*S*-Adenosylmethionine Decarboxylase) 68
  - C. *spe3* (Spermidine Synthase) 68
  - D. *spe4* and *SPE40* (Spermine Synthase) 68

III. Other Microorganisms 69
  - A. *Neurospora crassa* 69
  - B. *Aspergillus nidulans* 69
  - C. *Haemophilus parainfluenzae* 69

References 69

I. *ESCHERICHIA COLI*

Large amounts of putrescine and spermidine are present in *Escherichia coli*.[1] These amines constitute a large fraction of the acid-soluble ninhydrin-reactive material in *E. coli*. The biosynthetic pathway for these amines in *E. coli* is shown in Figure 1. In *E. coli* two pathways are used for the formation of putrescine; namely, ornithine decarboxylase or arginine decarboxylase plus agmatine ureohydrolase. The enzymes necessary for each step have been purified. For specific references, see the bibliography in Reference 1.

The gene for each step in the biosynthetic pathway has been mapped on the *E. coli* chromosome, and both point and deletion mutations have been described.[1] The gene designations for each step have been indicated in Figure 1. Since mutations in any one of these genes do not lead to striking changes in growth rate (see below), some of our genetic studies have been carried out by measuring the $^{14}CO_2$ formed from carboxyl-labeled arginine or ornithine. For this purpose a rapid technique was developed for screening large numbers of single clones.[2,3] Mutagenesis was usually carried out by nitrosoguanidine treatment for production of point mutations or by use of bacteriophage Mu for production of deletions; for the latter procedure, a technique was developed which permitted insertion of the bacteriophage Mu into specific areas of the chromosome.[4]

A. *speA* (Arginine Decarboxylase) and *speB* (Agmatine Ureohydrolase)

Putrescine biosynthesis from arginine requires the coupled action of arginine decarboxylase to form agmatine, and agmatine ureohydrolase to form putrescine and urea[5] (Figure 1). Both point and deletion mutations have been reported.[4,6-10] *speA* and *speB* are very closely linked and are located at 62.8 min on the chromosomal map; the gene order is *serA speB speA metK speC glc*.[11-13] Deletion mutations obtained with the bacteriophage Mu technique include both the *speA* and *speB* genes.

Plasmids have been constructed that contain both *speA* and *speB* or the *speA* gene separately. These plasmids have been used to overproduce arginine decarboxylase and agmatine ureohydrolase.[13-16]

B. *speC* (Ornithine Decarboxylase)

The *speC* gene is located at 63.4 min on the chromosomal map.[1,11-13] Both point mutations[11] and deletion mutations[4] have been described in this gene. We have also found that one mutant deleted in the *glc* locus (which maps at 64 min) is also deleted for *speC*.[12,17]

Plasmids have been constructed that contain the *speC* gene; these have been used to overproduce ornithine decarboxylase[13,14] and to isolate *specC* DNA.[18]

C. *speE speD* Operon (Spermidine Synthase and Adenosylmethionine Decarboxylase)

We originally isolated point and deletion mutations in *speD*,[4,19] which resulted in a loss of *S*-adenosylmethionine decarboxylase; the *speD* gene was mapped at 2.7 min on the *E. coli* chromosome. Recently[20] we showed that the strains containing a deletion in the *speD* gene also lacked spermidine synthase (putrescine aminopropyltransferase) which is coded for by the *speE* gene. The *speE* gene is immediately upstream to the *speD* gene. These two genes form an operon, since they are both controlled by a common upstream promoter. The mutation we previously designated as Δ*speD*[4,19,20] should now be defined as Δ(*speE speD*).

Plasmids have been constructed which contain *speE*, *speD*, or both *speE* and *speD*. These plasmids have been used for the overproduction of spermidine synthase and *S*-adenosylmethionine decarboxylase, and for obtaining the nucleotide sequence of the *speE speD* operon.[14,20-23] In these studies we also established that the *speE speD* operon represents the structural genes for these two enzymes and that *S*-adenosylmethionine decarboxylase is first synthesized as a proenzyme.[20,22,23]

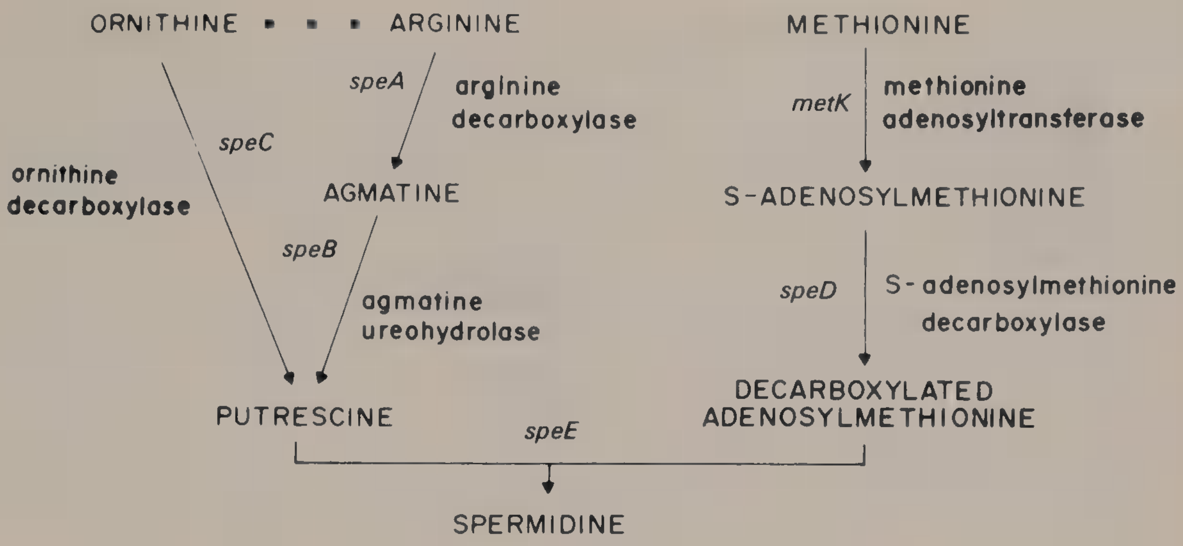

FIGURE 1. The biosynthetic pathway for putrescine and spermidine in *Escherichia coli*.

D. *cadA* (Lysine Decarboxylase)

The *cadA* gene is located at 92 min on the chromosomal map; the gene order is *mel cadA groE ampA purA*.[24] A regulatory gene (*cadR*) has also been described, located at 46 min on the chromosomal map.[24] Strains with the *cadR* mutation overproduce lysine decarboxylase. The *cadR* mutant is resistant to *S*-aminoethylcysteine (thiosine) and may be the same as *lysP*.[25]

Only point mutations have been described for *cadA*. A small residual lysine decarboxylase activity has been reported in such strains, which has been attributed to a weak constitutive lysine decarboxylase.[26] It should also be noted that lysine can serve as a poor substrate for ornithine decarboxylase.[27]

E. *metK* (Adenosylmethionine Synthetase)

Point mutations have been obtained in the *metK* gene; these mutants still have some residual enzymatic activity.[1,12,28-30] No deletion mutations have been observed; we assume that such mutants would be lethal in view of the general importance of adenosylmethionine to a variety of reactions. The *metK* gene maps at 63.1 min on the *E. coli* chromosome.[12,30]

Plasmids have been constructed that contain the *metK* gene.[13,14] These plasmids have been used to overproduce adenosylmethionine synthetase and for sequencing the gene.[31-33]

F. *pat* (Putrescine Aminotransferase)

A mutant with low levels of putrescine aminotransferase (10% of wild-type levels) has been isolated. This strain grows at 10% of the wild-type rate when arginine, ornithine, or putrescine is used as the nitrogen source. The *pat* gene is located at 89 min on the chromosome.[34]

G. Other *Escherichia coli* Enzymes

No mutants have been found for the biodegradative ornithine decarboxylase or the biodegradative arginine decarboxylase, or for the enzymes involved in the synthesis and breakdown of acetylputrescine, acetylspermidine, or glutathionylspermidine.[1] Mutants are known, however, in the biosynthesis of glutathione;[35] such mutants would, of course, be unable to make glutathionylspermidine.

H. Characteristics of Polyamine-Deficient *Escherichia coli*

The availability of deletion mutations in the genes of the biosynthetic pathway permits the construction of strains that are not able to make any polyamines and hence have no polyamines when grown in purified media. Such mutants are particularly suitable for studies on the physiological role of polyamines in vivo and have considerable advantages over the use of inhibitors for this purpose; most inhibitors produce only partial lowering of the polyamine levels and often have various side effects. The availability of such an in vivo test system is particularly desirable for studies on the physiological role of polyamines, since the polycationic character of the polyamines makes it very difficult to rule out nonspecific ionic interaction in in vitro studies.

To obtain strains that cannot make any polyamines, mutations must be present in several of the biosynthetic genes. Strains that lack only *speA*, *speB*, or *speC* still can make putrescine by using the remaining pathway. Thus, both *speA* (or *speB*) and *speC* mutations must be present to ensure inability to make putrescine.[8,10,11] (In some studies, indirect methods have been used to accomplish this purpose; thus, ornithine formation can be repressed by the presence of excess exogenous arginine, effectively eliminating ornithine as a source of putrescine.[8,11])

It is desirable to include the *cadA* mutation in the polyamine-deficient strains to exclude the formation of cadaverine by lysine decarboxylase; intracellular levels of cadaverine and its aminopropyl derivative increase in polyamine-deficient strains if the *cadA* mutation is not included.[24,27,36] Most of our studies have been carried out with Δ(*speA speB*) Δ*speC* Δ(*speE speD*) *cadA*, Δ(*speA speB*) Δ*speC*, or with Δ(*speA speB*) Δ*speC* Δ(*speE speD*) strains.[10,24,37-39]

Although the availability of the polyamine-deficient strains has not yet led to a definitive elucidation of the function of polyamines in vivo, a number of striking effects have been described. Strains that completely lack polyamines grow indefinitely, even in purified medium, but at a rate that is one third of the growth rate observed in the presence of amines.[10,24] Such strains are suitable hosts for wild-type bacteriophages T4 and T7 but are not suitable as hosts for bacteriophage λ. Defects have also been observed in Hfr recombination, as well as in the adsorption of male phages, possibly due to defects in the pili, if the bacteria are polyamine deficient.[10]

Of particular significance are observations indicating the effect of polyamines on protein biosynthesis in vivo. We have reported, for example, that polyamine-deficient strains show a marked decrease in growth rate if certain *rpsL* (*strA*) mutations are transduced into these strains; in some of these *rpsL* strains the requirement for polyamines is now absolute.[37] One of these *rpsL*-containing polyamine-deficient strains has a temperature-dependent requirement for polyamines, that is, the strain has an absolute requirement for polyamines at 42°C but not at 30°C.[38] Since *rpsL* mutations affect the structure of ribosomal protein S12, these data can be interpreted as indicating an effect of polyamines on protein biosynthesis, particularly on the ribosome containing protein-synthesizing complex. This postulation is supported by our other studies showing very poor multiplication of certain amber mutations of bacteriophage T4 and T7, but not of wild-type phage, in polyamine-deficient strains even in the presence of a suitable suppressor. In addition, in the latter experiments there was an increased accumulation of amber fragments in the absence of polyamines.[39] Studies from a number of other laboratories, using both in vivo and in vitro techniques, have also indicated an effect of polyamines on various aspects of protein biosynthesis. (For references, see bibliographies in Reference 1 and 40.)

Very little phenotypic effect is noted with *speE speD* mutations (which contain putrescine but not spermidine). The growth rate is decreased by only 15% when these strains are grown in polyamine-free media.[19]

II. *SACCHAROMYCES CEREVISIAE*

Saccharomyces cerevisiae contains large amounts of spermidine and low concentrations of putrescine and spermine.[1,41] The biosynthetic pathway (Figure 2) is very similar to that of *E. coli*; however, only one pathway (ornithine decarboxylase) is present for putrescine

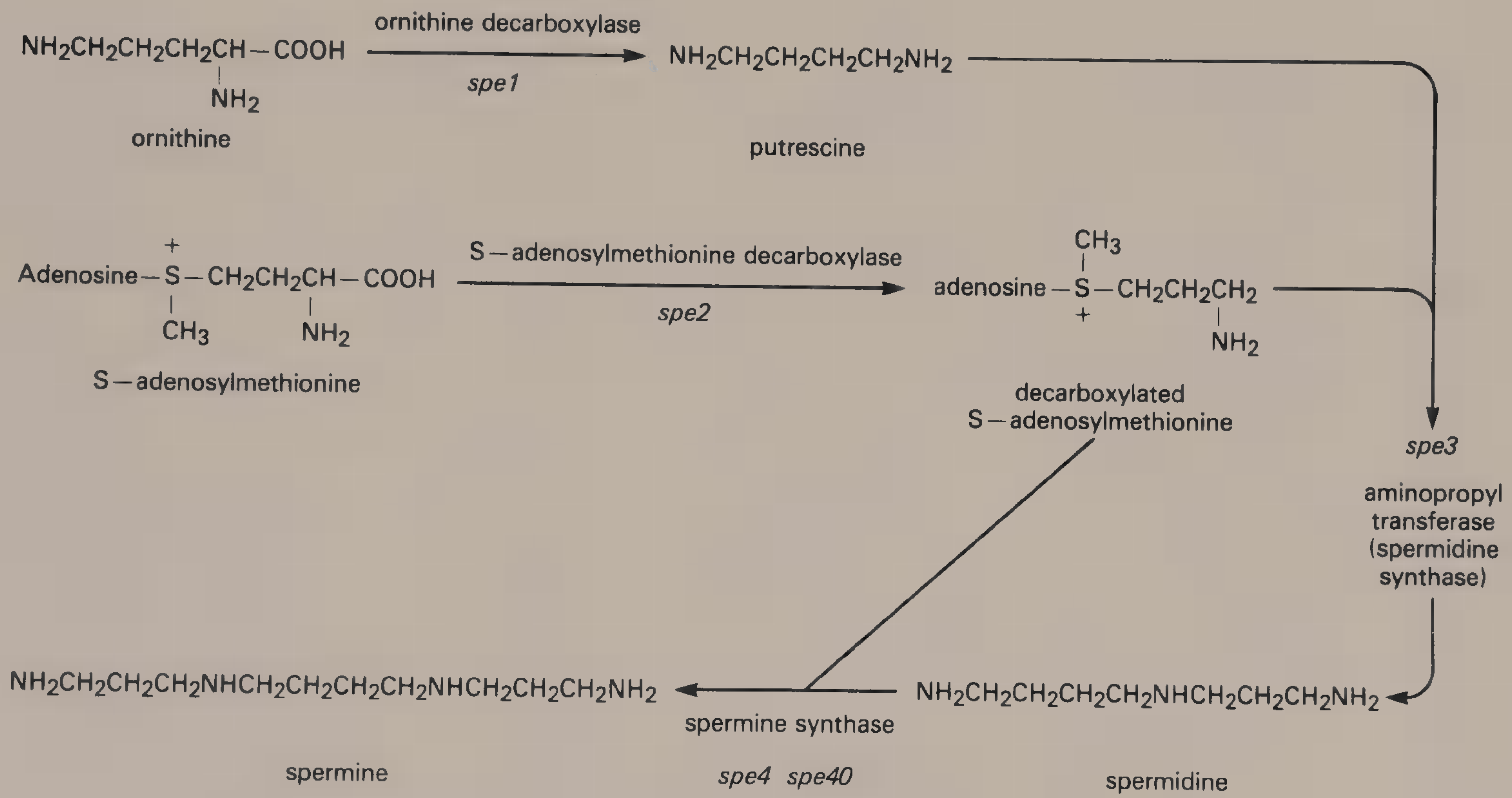

FIGURE 2. The biosynthetic pathway for putrescine, spermidine, and spermine in *Saccharomyces cerevisiae*.

biosynthesis, and two different aminopropyltransferases are required for spermidine synthesis and spermine synthesis.

A. *spe1* (Ornithine Decarboxylase)

With mutations in this gene, 13 independent isolates have been reported.[1,42-44] All of the mutations map in the same locus; the chromosomal location of this locus is not known.[45] Recently the *spe1* gene has been cloned and has been shown to be the structural gene for ornithine decarboxylase.[46,68*] (Our earlier studies had suggested that it was a regulatory gene.[42] For this reason we had previously referred to this gene as *spe10*. We now feel that this gene should be called *spe1*.)

Several of the mutants have an absolute requirement for added amines for growth. Amine concentrations as low as 10^{-8} to 10^{-10} M result in some growth of these strains. Therefore, in order to show the absolute requirement, special care must be taken to eliminate all potential sources of amines in the preparation of the culture media. In addition, a long period of growth in the amine-free medium is necessary in order to completely deplete the endogenous stores of polyamines.[42]

When plates containing such amine-deficient cultures are incubated for a week, ‘‘bypass’’ mutations arise. These strains are not revertants, but contain mutations (*spe4* and *SPE40*) in the last step of the biosynthetic pathway (see Section D below).

B. *spe2* (*S*-Adenosylmethionine Decarboxylase)

Strains with mutations in this gene have no detectable *S*-adenosylmethionine decarboxylase, no spermidine or spermine, markedly increased ornithine decarboxylase, and moderately increased putrescine. *spe2* mutants grow in polyamine-free medium at one sixth of the rate found in media supplemented with spermidine or spermine.[44,47,48] In the absence of spermidine or spermine, *spe2* cells cannot sporulate and cannot maintain the killer plasmids.[49,50]

The *spe2* gene is located on chromosome XV, very close to *arg1*.[51] The *spe2* gene has been cloned in a single-copy vector.[52]

C. *spe3* (Spermidine Synthase)

One mutant lacking spermidine synthase (putrescine aminopropyltransferase) has been found. This mutant has the same phenotype as that described for *spe2* (above), except for the presence of high concentrations of decarboxylated adenosylmethionine and of adenosylmethionine.[48]

D. *spe4* and *SPE40* (Spermine Synthase)

Two types of mutants (*spe4* and *SPE40*) were obtained that completely lack spermine synthase (spermidine aminopropyltransferase). These two genes are completely unlinked to each other — *spe4* is recessive and appears to be the structural gene for spermine synthase; *SPE40* is dominant and appears to be a regulatory gene. The chromosomal locations of these genes are not known.[1,41,42]

spe4 and *SPE40* have a striking interaction with the *spe1* gene; indeed, *spe4* and *SPE40* mutations were isolated as phenotypic reversions of *spe1* cultures that were grown on polyamine-free media. *spe1 SPE40* strains grow at a near-normal rate and have near-normal levels of ornithine decarboxylase; they contain both putrescine and spermidine, but not spermine. *spe1 spe4* strains are also able to grow, but at a low rate; no detectable ornithine decarboxylase or polyamines are present. *spe1* and *SPE40* are very tightly linked; the two genetic loci have not been separated.[1,41,42,45] This interesting and complex control mechanism involving *spe1*, *spe4*, and *SPE40* is still unexplained. A simple explanation would be that

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[*] The sequence of the gene for the *S. cerevisiae* ornithine decarboxylase was published recently.[68]

involving *spe1*, *spe4*, and *SPE40* is still unexplained. A simple explanation would be that *spe1* mutants are hypersensitive to repression by spermine; however, spermine added to *spe1 SPE40* mutants does not result in a level of ornithine decarboxylase as low as that noted in *spe1* mutants.[42]

No phenotypic changes have been observed with strains containing *spe4* mutations in a *spe1*$^+$ strain except for the complete absence of spermine and spermine synthase.

III. OTHER MICROORGANISMS

A. *Neurospora crassa*

In *Neurospora crassa*, as in *S. cerevisiae*, putrescine is synthesized only by ornithine decarboxylase. Two types of mutants have been described that cannot synthesize putrescine: (1) *aga* mutants lack arginase and thus cannot convert arginine to ornithine. When these cells are grown in the presence of arginine, ornithine biosynthesis is repressed; hence no putrescine can be made.[53] (2) *spe1* mutants lack ornithine decarboxylase and need exogenous polyamines for growth.[54-57] The *spe1* gene maps on linkage group V and is the structural gene for ornithine decarboxylase. Two of the mutants contain nonsense mutations.[57]

B. *Aspergillus nidulans*

In 1955, Sneath[58] first found an *Aspergillus nidulans* mutant with an absolute requirement for putrescine. This mutation (*puA*) was later shown to map in linkage group II and to cause a deficiency in ornithine decarboxylase.[1,59-63] Putrescine is much more effective than spermidine or spermine, since these amines are transported poorly in this strain.[64] As with *N. crassa* (above), putrescine auxotrophy also occurs in double mutants that are both blocked in ornithine biosynthesis and lack arginase.[55]

A transport mutant of *A. nidulans* has also been reported; this mutant (*spsA1*) has a more effective uptake system than the *puA* mutants for spermidine and spermine.[64]

C. *Haemophilus parainfluenzae*

In 1948, Herbst and Snell[65] showed that a natural isolate of *Haemophilus parainfluenzae* required putrescine for growth, and they later carried out extensive studies on the specificity of this requirement. There are also a number of other organisms that show a growth-stimulatory effect of polyamines, but, in general, it is not clear whether these effects are due to mutations in the biosynthetic pathway or, as in the case of *Neisseria* or halophilic organisms, to protection of the bacteria from lysis. (For references, see bibliography in References 66 and 67.)

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Chapter 5

REGULATION OF THE PUTRESCINE BIOSYNTHETIC GENES (*speA*, *speB*, *speC*) IN *ESCHERICHIA COLI*

Stephen M. Boyle, Jonathan M. Wright, C. Satishchandran, and Jagdeep Buch

TABLE OF CONTENTS

- I. Introduction 74
    - A. Scope of Article 74
    - B. Review of Regulation of Putrescine Synthesis in *Escherichia coli* 74
        - 1. Feedback Inhibition and Repression Effects 74
        - 2. K^+ and Nucleotide Effects 76
        - 3. Protein Activators and Inhibitors 76
        - 4. Pathway Selection 77
        - 5. Effects of cAMP on *speA*, *speB*, and *speC* Expression 77
- II. Expression of the Putrescine Biosynthetic Genes 77
    - A. Plasmids Bearing the *speA*, *speB*, and *speC* Genes 77
    - B. Expression of *spe* Genes 79
        - 1. In Vivo 79
            - a. Gene Fusion 79
            - b. Messenger RNA Levels of the *spe* Genes 79
        - 2. In Vitro 79
- III. Conclusions 80
- Acknowledgments 82
- Addendum 82
- References 82

I. INTRODUCTION

A. Scope of Article

All types of cells surveyed to date possess positively charged aliphatic cations called polyamines (e.g., putrescine, spermidine, and spermine). Polyamines bind to many of the negatively charged components of a cell. Although the physiological functions of polyamines is not well understood at the molecular level, numerous studies have shown that they are required for normal cell growth.[1,2] Evidence to date suggests that polyamines facilitate many of the intracellular processes involved in the production of enzymatic and structural components necessary for replication of the cell. Since the concentration of polyamines is highly regulated by cells, and because of their implied role(s) in cell growth, it is important to understand how their concentration is regulated. An approach our laboratory has taken to study regulation of polyamine levels in *Escherichia coli* has been to study the regulation of the genes encoding the enzymes involved in the synthesis of the polyamines. In collaboration with the Tabors and co-workers, we have used genetic engineering technology to isolate the genes encoding the putrescine biosynthetic enzymes of *E. coli*.[3,4]

In this article we review relevant portions of the literature dealing with the regulation of putrescine synthesis in *E. coli*. In addition, we summarize our own experiments dealing with the expression of the *speA*, *speB*, and *speC* genes encoding arginine decarboxylase (ADC), agmatine ureohydrolase (AUH), and ornithine decarboxylase (ODC). By focusing on how *E. coli* regulates the expression of the *spe* genes, we believe this approach will elucidate some of the regulatory mechanisms that ultimately modulate polyamine levels in vivo, and may lead to a clearer understanding of the function of polyamines in cell physiology.

B. Review of Regulation of Putrescine Synthesis in *Escherichia coli*

Brief but valuable overviews of the regulation of the putrescine biosynthetic enzymes in *E. coli* have appeared in reviews by Morris and Fillingame[5] and by Tabor and Tabor.[6] Two types of ODC and ADC are found in *E. coli*, a biodegradative and a biosynthetic form. The biodegradative ODC and ADC enzymes are inducible and their production favored in enriched growth media of high substrate concentration and acidic pH.[1] The biosynthetic enzymes are thought to be constitutive and their production favored in minimal growth media of neutral pH and low levels of substrates.[7,8] Both biodegradative ADC and ODC are found in only one out of ten *E. coli* strains and are thought to be recently evolved from the biosynthetic types.[9,10] By contrast, little was known about AUH in *E. coli* as it had not been systematically studied until our laboratory undertook its characterization.[11,12] We have found, however, no evidence for a biodegradative AUH. In this review we focus, therefore, on the regulation of the biosynthetic types as they, unlike the biodegradative types, are found in all strains of *E. coli*.

In order to present an overview of the current concepts of putrescine biosynthetic enzyme regulation in *E. coli*, it is necessary to define their relationships to their major products, putrescine and spermidine. As depicted in Figure 1, ornithine is an intermediate in the synthesis of arginine, and the amount of putrescine produced is regulated in an unknown fashion by the intracellular level of arginine. When *E. coli* is supplied with exogenous arginine, the intracellular ornithine pool decreases, as does the amount of putrescine produced from ornithine (pathway I). The existence of ADC and AUH provides an alternative means (pathway II) for the production of putrescine. Putrescine produced by either pathway I or II is converted to spermidine by spermidine synthetase.[6]

1. Feedback Inhibition and Repression Effects

Both putrescine and spermidine feedback inhibit ODC and ADC and this inhibition has been shown to be competitive in crude,[13] partially purified,[14] and purified preparations[15] of

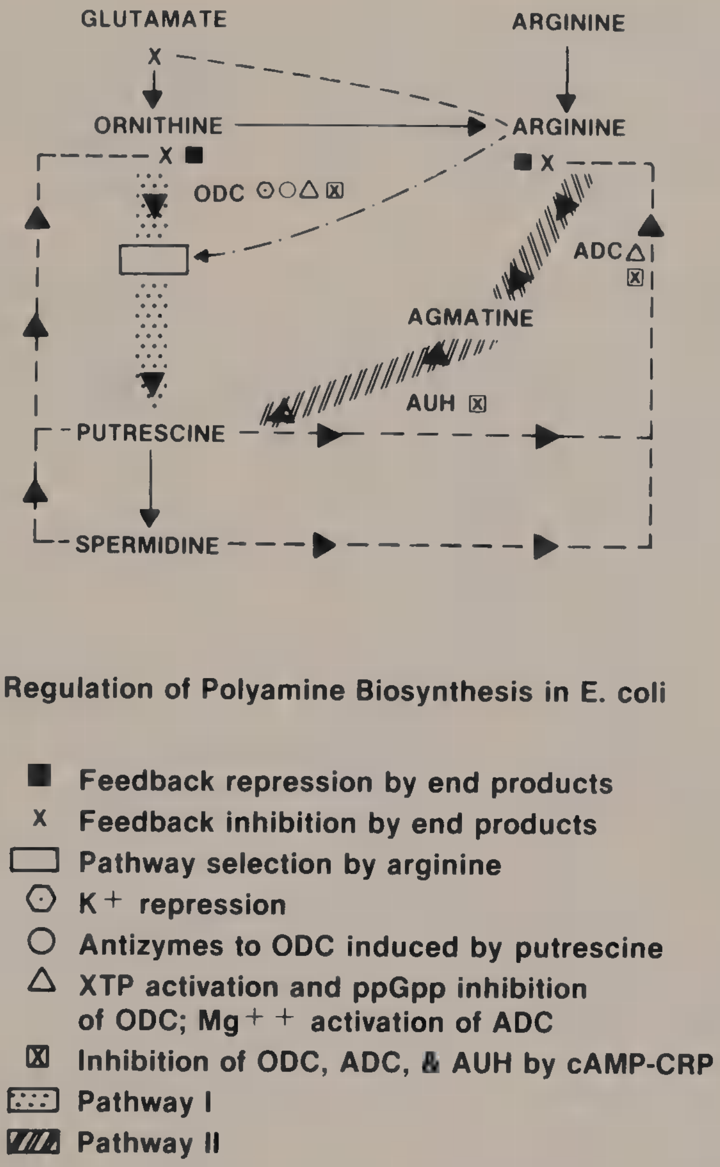
GLUTAMATE
ARGININE
ORNITHINE
ARGININE
ODC ⊙○△☒
ADC△
☒
AGMATINE
AUH ☒
PUTRESCINE
SPERMIDINE
Regulation of Polyamine Biosynthesis in E. coli
■ Feedback repression by end products
X Feedback inhibition by end products
▭ Pathway selection by arginine
⊙ K+ repression
○ Antizymes to ODC induced by putrescine
△ XTP activation and ppGpp inhibition of ODC; Mg++ activation of ADC
☒ Inhibition of ODC, ADC, & AUH by cAMP-CRP
⬚ Pathway I
▨ Pathway II

FIGURE 1. Proposed modes of regulation for biosynthesis in *E. coli*.

both enzymes. We have recently shown that AUH is not feedback inhibited by putrescine or spermidine but is noncompetitively inhibited by ornithine and induced by agmatine.[11] The concentrations of putrescine and spermidine used in these studies are similar to those found in *E. coli* (i.e., 5 to 30 m*M*), suggesting that polyamines may be effective in feedback inhibition of ODC and ADC in vivo. However, interpretations of the ability of the polyamines to participate in feedback inhibition are severely limited by the inability to assess the degree of binding of the cationic polyamines to other anionic intracellular components, i.e., to assess their effective concentration. Unfortunately, no studies have been published in which both cations have been tested simultaneously. This type of study would lend credence to their proposed roles in feedback inhibition as it has been demonstrated that the ratio of putrescine to spermidine varies as a function of the growth rate of *E. coli*.[16]

Addition of either putrescine or spermidine to cultures of polyamine-deficient *E. coli* causes partial repression of ODC and ADC activities.[13,14,17] In contrast, putrescine supplementation has no effect on AUH activity.[11] The conclusion drawn from these studies, that polyamines feedback repress ODC and ADC, relied on estimating levels of the enzymes based solely on their biological activity in crude extracts. Moreover, since the activities of the decarboxylases are sensitive to a variety of negative and positive effectors,[18,19] some of which are induced by polyamines, it is difficult to draw valid conclusions regarding the role of polyamines in *spe* gene expression. In order to accurately determine if repression of the putrescine biosynthetic enzymes occurs in vivo, levels of mRNA coding for ODC, ADC, and AUH and/or the amounts of the ODC, ADC, and AUH must be determined. These types of experiments are currently being conducted and preliminary results are described in Section II.B.2 and References 39 and 40.

2. K^+ and Nucleotide Effects

Initially a discrepancy existed between the Km of 5.6 m*M* for ornithine determined for a purified preparation of ODC and the intracellular level of ornithine estimated 100-fold below the apparent Km.[8,14] This discrepancy wa subsequently explained by the demonstration that certain nucleotide phosphates are positive effectors of ODC activity.[9,18] Guanosine triphosphate (GTP) was the most effective activator and at saturating concentrations, the Km for ornithine decreased to 0.28 m*M*. Activation of ODC activity by GTP influences both the Km and Vmax and, significantly, the GTP effect is reversed by putrescine.[9] A putative competition between GTP and putrescine for a site on the enzyme has been suggested and would partially account for the inhibition of ODC activity by putrescine. Neither ADC[9] nor AUH[12] is activated by nucleotides.

An inverse relationship exists between the intracellular putrescine levels in *E. coli* and the osmolality of the culture medium in which the cells are grown.[20] *E. coli* grown in culture media of high osmolality results in decreased putrescine levels due, in part, due to enhanced putrescine excretion.[20-22] In addition, utilizing a K^+ transport mutant of *E. coli*, it was demonstrated that replacement of the K^+ ions with Na^+ ions leads to a five- to eight-fold increase in putrescine production.[23] The degree to which osmotic regulatory mechanisms play in regulating ODC activity in *E. coli* is not known. By contrast, more recent experiments suggest that the polyamines themselves are not involved in osmoregulation. This conclusion was based on the observations that *E. coli* treated with monofluoromethylornithine (an irreversible inhibitor of ODC) resulted in a 60% reduction of putrescine and spermidine, yet the intracellular Na^+ and K^+ concentrations were unaffected.[24]

Partially purified preparations of ODC and ADC are competitively inhibited by guanosine tetraphosphate (ppGpp);[25] in contrast, purified AUH is unaffected by ppGpp.[12] Since amino-acid starvation inhibited polyamine accumulation in only stringent and not relaxed strains of *E. coli*, it was postulated that ppGpp regulates polyamine biosynthesis in *E. coli*.[25] However, experiments designed to test the regulation of ODC or ADC by ppGpp in vivo failed to substantiate a role for either GTP or ppGpp in *spe* gene expression.[26,27] It appears therefore, that a conflict exists as to whether or not ppGpp has a role in the regulation of these decarboxylases at a transcriptional and/or post-translational level. Studies with cloned copies of the *spe* genes will readily permit an assessment of whether ppGpp influences *spe* transcription. In addition, mutations in the consensus sequence for ppGpp[28] should alter *spe* expression in response to changes of ppGpp in vivo if ppGpp plays a transcriptional role.

3. Protein Activators and Inhibitors

Protein effectors of ODC and ADC have been found in *E. coli* cultured in enriched medium; effector activities are increased further following polyamine supplementation. One of the effectors, originally named ODC-antizyme,[19] appears to be at least three distinct proteins.[29] Two of these proteins inhibit the activity of ADC but at 10- to 30-fold less than the inhibition seen for ODC activity. The other effector is an ODC activator whose activity increases 1.6-fold in response to exogeneous polyamines. The ratio of activator units to inhibitor units in *E. coli* cultured in enriched medium drops from 50:1 to 3:1 following the addition of 5-m*M* putrescine and spermidine. Further studies are necessary to clarify the physiological function these effectors play in modulating ODC and ADC activities in cells cultured in minimal medium. Studies conducted in our laboratory indicate that there is very little if any ODC or ADC antizyme activity in *E. coli* cultured in minimal medium.[30] During the course of purifying AUH from *E. coli* cultured in enriched medium, we detected a protein which inhibits AUH activity.[12] It will be of interest to see if this protein is the same or analogous to the antizyme proteins. Based on partial amino acid sequences of the antizyme proteins, the antizymes appear to be ribosomal proteins.[31]

4. *Pathway Selection*

The synthesis of putrescine in *E. coli* via pathway I (ODC) of pathway II (ADC and AUH) is modulated by exogenous arginine which acts either directly or indirectly as a signal for derepression of pathway II and repression of pathway I. The addition of arginine to *E. coli* cultures leads to decreased production of ornithine production from glutamate due to feedback inhibition and repression of acetyl-glutamate synthetase by arginine.[32] Since ODC activity is reduced under these conditions, inherent in this explanation is that decreased ornithine or increased arginine levels lead to repression of ODC synthesis. In contrast to this scheme, ornithine supplementation leads to a decrease in the specific activity of ODC.[33] However, this latter study did not rule out the conversion of ornithine to arginine or putrescine which could be responsible for the inhibition of ODC.

Activation of pathway II by exogenously supplied arginine is not readily explained by mechanisms involving derepression, feedback inhibition, or substrate limitation.[14] The lack of evidence to support these specific regulatory mechanisms has led to the hypothesis that there is a specific channeling of exogenously supplied arginine into putrescine and that the pathway is less accessible to endogenously synthesized arginine.[14,34] A plausible explanation for the preferential access by ADC to the exogenous arginine would be for part of the ADC pool located in the periplasmic space. We have tested this prediction and found that ADC is synthesized with a signal sequence which would allow for its transport into the periplasmic space.[35] Thus, preferential utilization of pathway II in the presence of extracellular arginine is probably the result of arginine being decarboxylated in the periplasmic space.

5. *Effects of cAMP on speA, speB, and speC Expression*

We have demonstrated the cAMP negatively controls the expression of the genes encoding the putrescine biosynthetic enzymes.[11,30,36,40] In strains which bear mutations in either the adenylate cyclase gene (*cya*) or cyclic AMP (cAMP) receptor protein gene (*crp*), the activities of ODC, ADC, and AUH are derepressed 1.5- to 3.0-fold depending on the carbon source in which the strains are cultured. The addition of cAMP to cultures of wild type and *cya* strains causes 50 to 60% repression of these putrescine biosynthetic enzymes, whereas in cultures of *crp* strains no repressive effects were observed. We considered the possibility that the cAMP-mediated repression was indirect in that cAMP stimulated the production of either an as yet uncharacterized inhibitor of the putrescine biosynthetic enzyme activities or of a repressor of *speA*, *speB*, *speC* transcription. Summarized below are the results of experiments demonstrating the direct repression of *spe* gene transcription as a function of *spe* promoters by cAMP and its receptor protein.

II. EXPRESSION OF THE PUTRESCINE BIOSYNTHETIC GENES

A. Plasmids Bearing the *speA*, *speB*, *speC* Genes

The *spe* genes encoding ODC, ADC, and AUH were originally isolated from the Clarke-Carbon collection of hybrid ColE1 plasmids.[3] The expression of the *spe* genes borne on the plasmid vector pBR322 have been characterized with respect to enzyme activities of transformed *E. coli spe* deletion mutants and the molecular weights of the putrescine biosynthetic enzymes synthesized in minicells transformed with these plasmids.[4] In Figure 2, the restriction maps of pODC (ODC) and pKA5 (ADC and AUH) are presented in order to

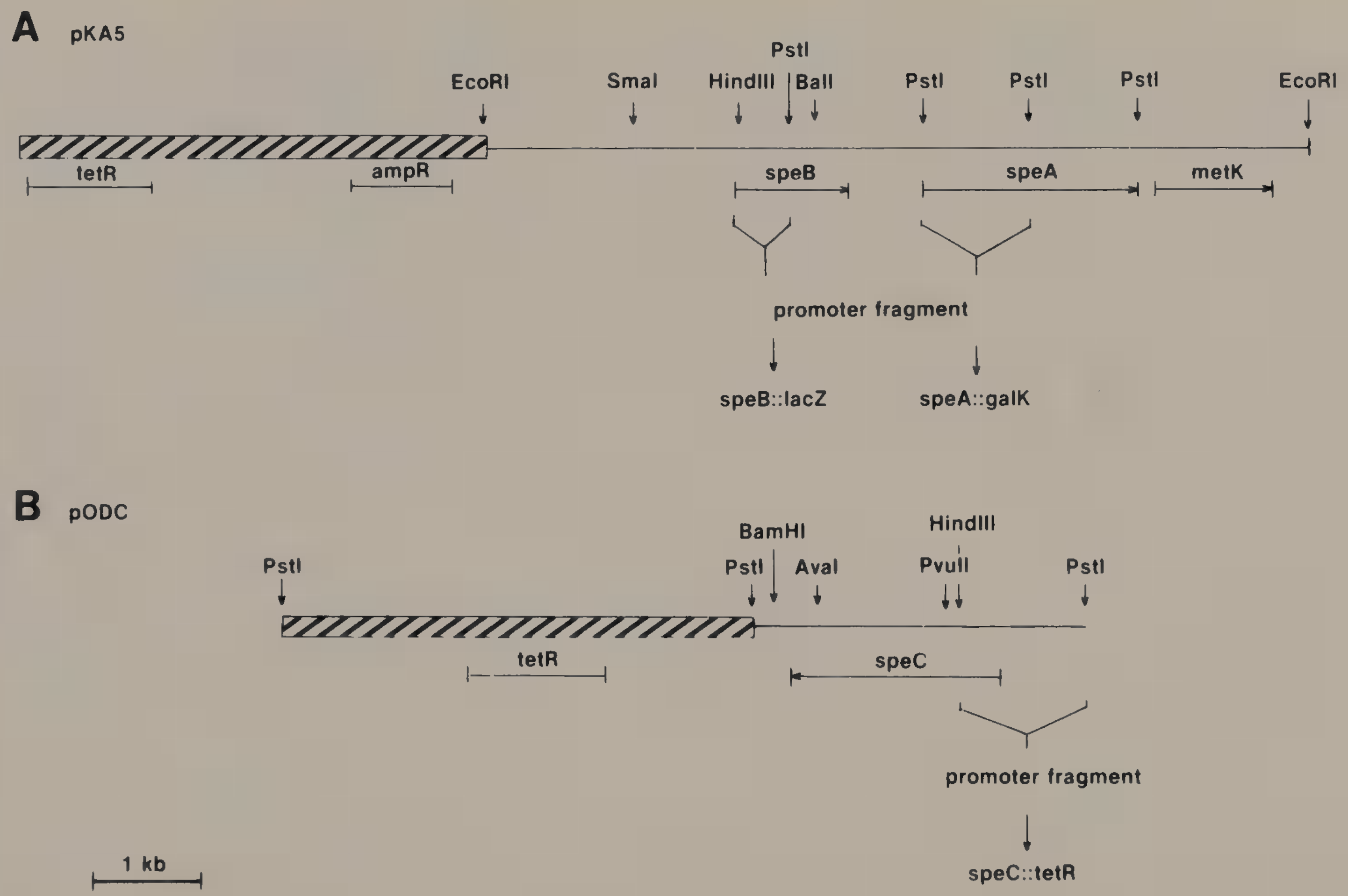

FIGURE 2. Restriction endonuclease sites, promoter regions, and structural genes of pODC and pKA5 plasmids.

demonstrate the size of the genes and the locations of the fragments bearing the promoter regions described in the following experiments.

B. Expression of the *spe* Genes

1. In Vivo

a. Gene Fusion

If cAMP and the cAMP receptor protein (CRP) repress transcription of the *speA*, *speB*, and *speC* genes, then any promoterless structural gene fused to a *spe* promoter should be repressed by the cAMP-CRP complex.

When a 1-Kb region containing the *speC* promoter (see Figure 2) was fused to the tetracycline resistance gene (*tetR*), addition of 5 m*M* cAMP to cultures of strains transformed with a plasmid bearing this hybrid *speC*::*tetR* gene resulted in decreased tetracycline resistance, but only in wild-type *E. coli* or a *cya* strain and not in a *crp* strain.[36,37] As a control, we demonstrated that the expression of the *tetR* gene fused to its own promoter was not inhibited by the addition of cAMP in any of these strains.

When a 0.8-Kb fragment of the *speB* gene from pKA5 (see Figure 2) was fused to the promoterless *lacZ* gene (β-galactosidase) carried on the plasmid pMC1403, we found that concentrations of 1 to 10 m*M* cAMP caused a progressive inhibition of β-galactosidase expression in a wild-type *E. coli* or an isogenic *cya* strain bearing this fusion *speB*::*lacZ*. However, in an isogenic *cya*, *crp* strain bearing the *speB*::*lacZ* fusion, β-galactosidase expression was unaffected by the addition of cAMP.[39] As a control, we demonstrated that cAMP had no effect on penicillinase expression encoded by the plasmid in wild type, *cya* or *cya*, *crp* strains of *E. coli*.

Similarly, a 0.9-Kb fragment containing the *speA* promoter and derived from pKA5 (see Figure 2) was fused to the promoterless *galK* gene (galactokinase) or *tetR* gene. Wild-type *E. coli* or a *cya* strain transformed with plasmids bearing either *speA*::*tetR* or *speA*::*galK* fusions exhibited decreased tetracycline resistance or galactokinase expression when cultured in the presence of 1 to 10 m*M* cAMP. In contrast, an otherwise isogenic *crp* strain transformed with either plasmid bearing *speA*::*tetR* or *speA*::*galK* fusions exhibited no change in tetracycline resistance or galactokinase expression when cultured in the presence of cAMP.[38] Based on these results in vivo in which the promoters of the *speA*, *speB*, and *speC* genes were fused to a number of promoterless structural genes, we conclude that the *spe* promoters are responsible for the repression mediated by the cAMP-CRP complex.

***b. Messenger RNA Levels of the spe* Genes**

Although our analysis of *spe* gene expression in vivo using the promoter fusion constructs has provided compelling evidence for negative control by cAMP and CRP, it is not a direct measurement of *spe* transcription. Thus, in order to corroborate this effect as a transcriptional one, we measured the amount of various *spe* messenger RNAs in wild-type, *cya*, and *crp* strains of *E. coli*. Utilizing the quick-blot hybridization methodology, we have measured the expression of the *speA*, *speB*, and *speC* genes in *E. coli*.[36,39,40] As shown in Table 1, the steady-state levels of mRNA encoding either ADC, AUH, or ODC were repressed approximately 80 to 90% by the addition of cAMP to a *cya* strain but not to a *crp* strain. In contrast, the level of mRNA (on the same blot) encoding β-galactosidase was induced 2.6-fold in the *cya* strain and unaffected in the *crp* strain by the addition of cAMP. These results indicate that the cAMP-CRP complex exerts negative transcriptional control of the *speA*, *speB*, or *speC* genes.

2. In Vitro

We considered the possibility that the repression of *spe* gene transcription observed upon the addition of cAMP to cultures of *E. coli* is an indirect one. This could be the result of

Table I

STEADY-STATE LEVELS OF *speA*, *speB*, AND *speC* mRNA IN *ESCHERICHIA COLI* STRAINS

	speA		*speB*		*speC*		*lacZ*	
Supplement	**cya**	**crp**	**cya**	**crp**	**cya**	**crp**	**cya**	**crp**
Control	100	100	100	100	100	100	ND	ND
IPTG	97	112	92	99	98	90	100	100
IPTG + cAMP	7	89	9	102	16	93	261	119

Note: *E. coli* strains LS853(cya) and LS854 (crp) were cultured in MOPS-glucose medium[30] in the presence or absence of 0.5-mm IPTG and 5-mm cAMP for five to six generations. The mRNA levels were measured using the quick-blot procedure.[37] Values are expressed relative to controls (100) in terms of percent change. ND means not detected. The results are the data averaged from three separate experiments and the SD averaged ± 9%.

cAMP-CRP complex inducing the synthesis of a repressor protein which in turn binds to the *speA*, *speB*, and *speC* genes to inhibit their transcription. Evidence to date argues against this possibility. Experiments using a coupled transcription-translation system directed by either the *speA*, *speB*, or *speC* genes have shown that the cAMP represses the synthesis of ADC, AUH, and ODC, respectively, only in the presence of a CRP protein.[36,39,40] Moreover, in *E. coli* (chromosomeless) minicells bearing the pODC plasmid, the addition of cAMP represses ODC synthesis but not β-lactamase synthesis encoded by pBR322.[36] In the case of minicells bearing pKA5 we have also seen repression of AUH by cAMP. Taken together these results clearly demonstrate that the cAMP-mediated repression of ODC, ADC, and AUH synthesis is exerted directly at the promoters of their respective genes.

III. CONCLUSIONS

Using various approaches both in vivo and in vitro we have demonstrated that the cAMP-CRP complex negatively regulates the transcription of the *speA* (ADC), *speB* (AUH), and *speC* (ODC) genes. The negative regulation of putrescine synthesis by cAMP seems extreme in light of the other mechanisms thought to be operative in *E. coli*. However, many of the previously postulated regulatory mechanisms have not been stringently tested and relied mostly on measuring changes in putrescine biosynthetic enzyme activities in vitro. The availability of cloned copies of the putrescine biosynthetic genes as well as promoter fusions (*speC*::*tetR*, *speB*::*lacZ*, *speA*::*galK*) will allow further in-depth examinations to determine if other regulatory mechanisms are operative in vivo. For example, we have found that putrescine represses the tetracycline resistance of strains bearing the *speC*::*tetR* fusion.[40] Furthermore, in a coupled transcription-translation studies, we found that putrescine inhibits ODC synthesis.[40] These observations are consistent with the feedback repression of polyamine biosynthetic genes postulated by Tabor and Tabor.[13] What is not known, however, is whether feedback repression is a direct mechanism in which putrescine interacts directly with the DNA in the *speC* promoter or interacts indirectly by a binding to regulatory protein which binds to the *speC* promoter. It will be of particular interest to learn whether cAMP and/or spermidine inhibits the synthesis of putrescine aminopropyl transferase (spermidine synthase) in *E. coli* as the ratio of putrescine and spermidine, although constant during steady-state growth, increases with increasing growth rate.[16] If the ratio of putrescine to spermidine is controlled predominantly by transcriptional regulation of the *spe* genes, one might expect differential regulation in which the promoters of the putrescine biosynthetic genes respond differently to various *trans*-acting regulatory factors, such as the cAMP-CRP complex, than does the promoter of the gene-encoding spermidine synthase.

Until relatively recently, it was believed that cAMP-CRP acts solely as an activator of catabolite-sensitive operons. The finding that the cAMP-CRP complex exerts negative transcriptional control of the *spe* genes as well as negative transcriptional control of *galP2*,[41] spot 42 RNA,[42] possibly *ompA*[43] *cya*,[44] and autogenous regulation of the *crp* gene[45] extends the role of the cAMP-CRP complex in prokaryotic gene regulation.

It is of particular interest that the level of cAMP regulates the production of polyamine molecules that are thought to be intimately involved in the control of macromolecular synthesis. The level of cAMP varies inversely with the growth rate of *E. coli* cells. Cells cultured on glycerol exhibit high intracellular levels of cAMP, whereas cells cultured on glucose, which allows a faster growth rate, have reduced intracellular levels of cAMP.[46,48] The rates of synthesis of DNA, RNA, and protein are markedly increased in rapidly growing cells.[49] If polyamines are required to facilitate macromolecular synthesis, it follows that the levels of these compounds would be elevated in rapidly growing cells.

cAMP coordinates the mobilization of potential reserves of carbon and energy when readily available sources become limiting. As a result, cAMP has been called an ‘‘alarmone’’ or ‘‘hunger signal’’, sensing the metabolic state of the cell. Similarly, in liver and muscle cells of mammals, cAMP acts as a hunger signal; cAMP transmits the signal in these cells via a protein kinase rather than by enhancing the transcription of catabolite-sensitive operons. The role of cAMP may be more extensive in prokaryotic physiology. Sanwal[50] reported that spermidine can replace magnesium as an activator of *E. coli* glucose-6-phosphate dehydrogense and may well be the natural activator of this enzyme. The ribose of RNA in *E. coli* is derived mainly from the pentose phosphate pathway, of which glucose-6-phosphate dehydrogenase is a key enzyme. Cohen[51] suggested that spermidine, a compound thought to facilitate RNA synthesis at the level of transcription, may also stimulate the supply of RNA precursors. The finding the cAMP negatively controls the production of spermidine via the putrescine biosynthetic enzymes as well as glucose-6-phosphate dehydrogenase[10,40] would be consistent wtih this scheme.

The repression of the tetracycline resistance, galactokinase, and β-galactosidase in *E. coli* *crp*$^+$ strains bearing the appropriate fusion genes by polyamines suggests that these cations negatively regulate *spe* transcription. This idea is supported by the finding that low concentrations of polyamines repress the synthesis of ODC in a cell-free transcription-translation system programmed with the *speC* serving as the template. There are several possible mechanisms for negative control of *speC* transcription by polyamines:

1. These cations may intercalate directly at the *speC* promoter to reduce initiation of transcription.
2. They may interact with RNA polymerase to specifically reduce initiation or elongation of transcription of the *speC* gene.
3. Polyamines may be ligands for a *speC* repressor. Since evidence summarized here indicates that CRP interacts with the *speC* promoter, this transcriptional regulator is a candidate for such a role. This hypothesis could be tested experimentally by examining the effect of polyamines on *speC* mRNA levels in a *crp* deletion strain.
4. Several reports indicate that the expression of certain genes in *E. coli* are regulated by the extenty of DNA supercoiling.[52,54] The polyamines stimulate DNA gyrase activity, and inhibit the activity of topoisomerase I in vitro.[55,56] Thus, polyamines have the potential to increase DNA supercoiling in vivo. An increase in negative superhelicity of the chromosome may cause local changes in the region of tjhe *speC* promoter leading to decreased initiation of transcription. Examining the effect of polyamines on ODC mRNA levels in *E. coli* strains with defective topoiosmerase activities would test this hypothesis. Also, the effect of polyamines on *speC* transcription in a cell-free system directed by linerarized pODC-1 could be examined.

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ADDENDUM

A. Antizymes as Regulators of ODC

Kashiwagi and Igarashi[57] have demonstrated that in addition to the specific ribosomal proteins S20/L26 and L34, all of the additional ribosomal proteins with the exception of 12 exhibit ODC-antizyme activity. Moreover, when the ribosomal proteins were part of the ribosome (i.e., complexed with ribosomal RNA), no ODC-antizyme activity was detectable. They also demonstrated that both synthetic and naturally occurring basic polypeptides exhibited ODC antizyme activity. As the pools of ribosomal proteins in *E. coli* are very small and ribosomes seldom undergo rapid breakdown, the possibility that ribosomal proteins act as regulators of ODC activity in vivo appears to be marginal.

B. Polyamine Levels in *Escherichia coli*

Additional studies by Kashiwagi and Igarashi[58] have shown that *E. coli* containing a plasmid-born *speC* gene produces excess putrescine which is excreted into the culture medium. Of note is that the intracellular putrescine and spermidine in *E. coli* with a plasmid-born copy of the *specC* gene is the same as that in the strain bearing a single copy of the *speC* gene. Thus, *E. coli* appears to have a very efficient means to regulate intracellular putrescine; moreover, this mechanism appears to be independent of the carrier protein identified to be responsible for putrescine uptake in *E. coli*.[59]

C. *speB::galK* Gene Fusion

Unpublished studies in our laboratory have demonstrated that the *speB::galK* fusion discussed earlier[39] is not a fusion of the promoter region of *speB* to the *galK* gene. It appears that the hybrid gene represents a fusion of a promoter on the complimentary strand of *speB* that is regulated by the cAMP-CRP complex. Thus it is possible that *specB* is negatively regulated by a transcript (e.g., antisense mRNA) or an as yet unidentified protein encoded by a gene on the complimentary strand of the *speB* gene. Experiments are in progress to determine the nature of *speB* regulation with respect to cAMP.

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Chapter 6

UTILIZATION OF L-ARGININE, L-ORNITHINE, AGMATINE, AND PUTRESCINE AS THE MAJOR SOURCE OF NITROGEN AND ITS CONTROL IN *ESCHERICHIA COLI* K-12

Yeheskel S. Halpern and Esther Metzer

TABLE OF CONTENTS

I. Introduction 86

II. Elucidation of the Pathway for the Utilization of Arginine and Ornithine as a Sole Nitrogen Source by *Escherichia Coli* K-12 87
  A. Transduction of *speA speB* into Strain CS101B 88
  B. Transduction of *speB* into Strain CS101B 88
  C. Transduction of *speC* into Strain CS101B 88
  D. Isolation and Characterization of a CS101B Pat$^-$ Mutant 89
  E. Isolation and Characterization of a CS101B Prr$^-$ Mutant 90

III. Regulation of Enzyme Synthesis in the Arginine/Ornithine Catabolic Pathway 90
  A. Catabolite Repression 90
  B. Effect of cAMP 90
  C. Control by the *ntr* Regulatory System 91
  D. Induction 91

Acknowledgment 94

References 94

I. INTRODUCTION

Bacterial metabolism of arginine, agmatine, ornithine, and putrescine proceeds by several different pathways. In *Bacillus subtilis,*[1] *Proteus vulgaris,*[2] and yeast,[3] arginine is degraded to ornithine and urea. The ornithine is metabolized to glutamate and the urea is hydrolyzed to CO_2 and NH_3. *Klebsiella aerogenes*[4,5] also breaks down arginine to ornithine, but without urea being released in the process. According to Friedrich and Magasanik,[4] an amidinotransferase catalyzes the transfer of the guanido group of arginine to an unknown acceptor. Cunin et al.[6] maintain that in *K. aerogenes* arginine is mainly metabolized via the arginine succinyltransferase pathway. Arginine utilization by the combined action of arginine decarboxylase (ADC) and agmatinase (AUH) is essentially directed towards polyamine biosynthesis.

Many pseudomonads[7] and clostridia[8] degrade arginine to citrulline and ammonia; the citrulline is converted to ornithine which is catabolized to glutamate. In *Escherichia coli* W[9] *Pseudomonas putida*[10] and *Streptomyces griseus,*[11] arginine is metabolized to succinate via putrescine and γ-aminobutyric acid (GABA).

Studies from this laboratory[12,13] clearly showed that the utilization of arginine and ornithine as the sole source of nitrogen in *E. coli* K-12 is by way of putrescine, which is degraded to succinate via GABA, as shown in Figure 1.

Nitrogenous compounds, such as arginine, ornithine, and putrescine can be utilized by the bacterial cell as both a carbon and nitrogen source. The utilization of carbon sources is widely controlled by catabolite repression.[14] In catabolite repression-sensitive systems, enzyme synthesis is repressed in a glucose minimal medium and derepressed upon growth on a poorer carbon source such as succinate. In the catabolism of a compound that can provide both the carbon and nitrogen requirements of the cell, catabolite repression could be deleterious, as described by Prival and Magasanik,[15] in *Salmonella*. When grown in a glucose-histidine medium, *Salmonella* is unable to utilize the nitrogen of histidine due to catabolite repression of the histidine utilization (*hut*) enzymes. In *Klebsiella* and *E. coli*, catabolite repression of the synthesis of enzymes catalyzing the utilization of nitrogenous carbon compounds is modulated by the nitrogen supply. When nitrogen supply is limiting the catabolite repression of these enzymes by glucose is relieved. The role of the *gln* regulatory genes in this dual control has been established both in *Klebsiella*[16-18] and in *E. coli*.[19] Thus, a *gln* mutation which prevents glutamine synthetase (GS) formation and results in a glutamine requirement, also prevents derepression of the *hut* system in *Klebsiella*[17] and of the GABA degradation (*gab*) system in *E. coli*,[19] when nitrogen supply is limited. *gln* mutations, which bring about derepressed synthesis of GS in a glucose medium even in the presence of an abundant supply of nitrogen, also result in the derepressed synthesis of the *hut* and *gab* enzymes, regardless of the amount of nitrogen available.

We have recently shown that the enzymes AUH, putrescine aminotransferase (PAT), and pyrroline dehydrogenase (PrrD), which catalyze the utilization of arginine, ornithine and putrescine as a source of nitrogen in *E coli*, are regulated by catabolite repression and nitrogen availability.[12,13] The catabolite represssion of AUH can be fully relieved by the addition of exogenous cAMP, while PAT and PrrD, like the GABA enzymes,[20] are much less sensitive to the addition of cyclic adenosine-3′,5′-monophosphate (cAMP) to the growth medium.[12,13]

Since arginine, orthinine, agmatine, and putrescine are not usually abundant compounds, it is reasonable to expect that substrate induction and/or end-product inhibition would be part of the control network employed by the cell to regulate their utilization. In *Klebsiella*, substrate induction is important in the arginine and agmatine degradative pathways.[4,5] In contrast, the GABA-degradative pathway of *E. coli* K-12 is not an inducible system.[21,22] In recent work from this laboratory we have shown that the synthesis of AUH is stimulated

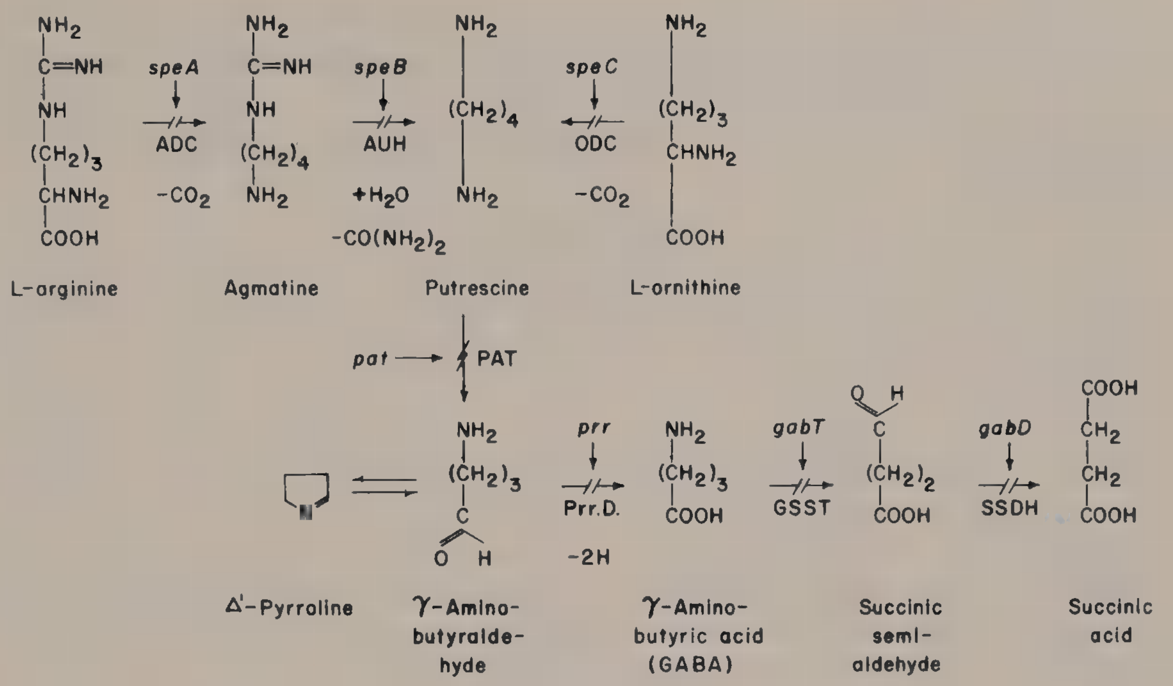

FIGURE 1. The degradative pathway of arginine and ornithine in *E. coli* K-12. ADC, L-arginine decarboxylase; AUH, agmatine ureohydrolase; ODC, L-ornithine decarboxylase; PAT, putrescine aminotransferase; Prr. D., Δ'-pyrroline dehydrogenase; GSST, GABA aminotransferase; and SSDH, succinic semialdehyde dehydrogenase. Mutant designations: *speA*, ADC$^-$ (5% of wild-type activity or less), arginine nonutilizing mutant; *speB*, AUH$^-$ (5% of wild-type activity), arginine and agmatine nonutilizing mutant; *speC*, ODC$^-$ (10% of wild-type activity), ornithine nonutilizing mutant; *pat*, PAT-defective (10% of wild-type activity or less), arginine, ornithine, and putrescine nonutilizing mutant; *prr*, Prr.D.-defective (approximately 10% of wild-type activity), pyrrolidine nonutilizing mutant; *gabT*, GSST-defective, GABA nonutilizing mutant (17); and *gabD*, SSDH$^-$ mutant.[17]

severalfold by arginine and agmatine, while PAT is only slightly induced, if at all, by putrescine. PrrD, like the GABA enzymes, is not substrate induced. The synthesis of ADC and ODC is not induced by substrate or repressed by glucose, nor is it stimulated by nitrogen limitation.[12,13]

II. ELUCIDATION OF THE PATHWAY FOR THE UTILIZATION OF ARGININE AND ORNITHINE AS A SOLE NITROGEN SOURCE BY *ESCHERICHIA COLI* K-12

The possibility that arginine is utilized at the 5-carbon level via ornithine as in *K. aerogenes*[4,5] was ruled out on the following evidence:

1. Amidinotransferase activity in extracts of arginine-grown *E. coli* K-12 CS101B was only about 10% of that found in *K. aerogenes*.
2. Strains M-16 and M-20, two GABA-nonutilizing mutants of *E. coli* K-12 CS101B grow on arginine and ornithine at rates similr to those of the parent CS101B strain, although the activity of ornithine aminotransferase, the second enzyme of the *Klebsiella* pathway, is very low in these mutants.
3. Introduction of a *putA* mutation, abolishing the activity of pyrroline-5-carboxylate dehydrogenase, the presumptive third enzyme of the *Klebsiella* pathway, into strain CS101B did not affect growth on arginine and ornithine.

An alternative pathway for the utilization of arginine and ornithine is degradation to putrescine via ADC and AUH and via ornithine decarboxylase (ODC), respectively. These reactions are part of the biosynthetic pathway of the polyamines in *E. coli*.[23] To see whether this is the operative pathway for the utilization of arginine and ornithine as the nitrogen source, we transduced strain CS101B with phage P1 lysates of mutants with lesions in the *speA,B,C* genes, specifying the synthesis of ADC, AUH, and ODC, respectively. Arginine-nonutilizing and ornithine-nonutilizing transductants were isolated after three cycles of penicillin selection.

A. Transduction of *speA speB* into Strain CS101B

Using a lysate of the *speA speB* double mutant EWH319 (ATCC No. 33232) we obtained 521 arginine-nonutilizing colonies that grew well on ornithine, putrescine, GABA, and proline as the nitrogen source. Ten of these colonies picked at random were ADC$^-$ and AUH$^-$. One such transductant, strain CS101B319, was transduced with a lysate of a Met$^+$ derivative of strain CS101B and arginine utilizing transductants appeared at a frequency of 5×10^{-6}, similar to the frequency of Met$^+$ transductants (3.7×10^{-6}). Ten randomly picked arginine-utilizing transductants were all found to have regained wild-type ADC and AUH activities. Reciprocal transductions between strains CS101B319 and EWH319 yielded no arginine-utilizing recombinants. *speA* was separated from *speB* by transducing strain 101B319 with phage P1 grown on CS101B Met$^+$ and selecting for growth on agmatine as the nitrogen source. Out of 585 agmatine-utilizing transductants 2 were unable to utilize arginine; they showed wild-type levels of AUH, but negligible ADC activity (CS101b3191).

B. Transduction of *speB* into Strain CS101B

Phage P1 grown on strain MA197(*speB*) was used to transduce strain CS101B. Arginine-nonutilizing strains were isolated by three cycles of penicillin selection. Out of 100 such colonies 2 could grow on ornithine, putrescine, GABA, and proline and were AUH$^-$. Transduction of one of them (strain CS101B197) with a CS101B Met$^+$ lysate yielded 2×10^{-5} arginine-utilizing transductants (as compared to 2.5×10^{-5} Met$^+$ transductants). Ten randomly chosen arginine-utilizing transductants were all shown to have regained wild-type AUH activity. Reciprocal crosses between MA197 and CS101B197 yielded no arginine-utilizing transductants.

C. Transduction of *speC* into Strain CS101B

A P1 lysate of strain MA255 (*speC*) Met$^+$ was used as donor and ornithine-nonutilizing transductants were isolated after three cycles of penicillin selection. Of 100 such colonies, 1 was able to grown normally on arginine, putrescine, GABA, and proline and had about 10% of the wild-type activity of ODC (strain CS101B255). The capacity for ornithine utilization could be transduced into stain CS101B255 by a P1 CS101B lysate. Ten of these ornithine-utilizing colonies were all shown to have regained wild-type ODC activity. Reciprocal crosses between strains CS101B255 and MA255 yielded no ornithine-utilizing transductants. Table 1 shows the relevant enzyme activities of the different transductants. The growth rates of these transductants on NH_4^+, arginine, agmatine, ornithine, putrescine, and GABA, as the sole nitrogen source, are given in Table 2. We also isolated two UV-induced mutants of strain CS101B, strains A-15 and A-18, that lost the ability to grow on arginine as the nitrogen source but could grown on ornithine, putrescine, GABA, proline and NH_4^+. These strains exhibited less than 10% of wild-type level of ADC activity. No arginine-utilizing transductants were obtained from crosses between A-15 or A-18 and the *speA* strain CS101B3191. In a similar fashion we isolated three arginine/agmatine nonutilizing mutants which grew well on the other nitrogen sources and showed low levels of AUH. Likewise, an ornithine nonutilizing mutant that had only 15% of the wild-type level of ODC was isolated. No ornithine-utilizing recombinants were obtained from crosses between this ODC$^-$ mutant and the *speC* strain CS101B255. It is clear from these data that transductants lacking ADC activity are unable to utilize arginine as the nitrogen source; cells lacking AUH

Table 1
ENZYME ACTIVITIES IN WILD-TYPE AND MUTANT STRAINS[a]

	Specific activity (nmol/min/mg protein)							
Strain	**ADC**	**AUH**	**ODC**	**PAT**	**PrrD**	**OAT**	**GSST**	**SSDH**
CS101A	185	220	170	17	960	23	25	17
CS101B	195	190	162	17	975	25	380	850
M16	210	190	210	15	NT[b]	2	80	0
M20	200	190	176	4	150	2	80	675
CS101B3191(*speA*)	6	185	150	16	NT	25	414	800
CS101B197(*speB*)	200	10	160	17	NT	24	401	800
CS101B319 (*speA,B*)	10	10	190	15	NT	28	380	675
CS101B255 (*speC*)	190	171	19	13	NT	21	415	850
CS101B Pat−	185	180	210	2	NT	18	284	574
CS101B Prr−	207	154	210	26	120	NT	309	424

[a] Cells were grown in succinate (1%)-ammonia (0.1%) minimal medium.

[b] NT, not tested.

Table 2
GROWTH RATES OF WILD-TYPE AND MUTANT STRAINS ON DIFFERENT SOURCES OF NITROGEN[a,b]

	Nitrogen source					
Strain	**NH_4^+**	**L-Arginine**	**Agmatine**	**L-Ornithine**	**Putrescine**	**GABA**
CS101A	90	162	150	160	150	360
CS101B	90	160	160	170	150	120
CS101B3191(*speA*)	90	420	160	130	140	170
CS101B197(*speB*)	95	300	300	165	160	120
CS101B319(*speAspeB*)	90	440	400	180	180	130
CS101B255 (*speC*)	90	170	160	600	180	130
CS101B Pat−	100	300	300	360	360	180
M16	100	175	NT[c]	175	210	360
M20	90	180	NT[c]	200	180	420

[a] Glycerol (0.5%), served as the carbon source. Nitrogen sources were added at 0.2%, except for $(NH_4)_2SO_4$, at 0.1%.

[b] Doubling time in minutes.

[c] NT, not tested.

are impaired in the utilization of both arginine and agmatine; and cells with no ODC activity cannot utilize ornithine.

D. Isolation and Characterization of a CS101B Pat$^-$ Mutant

A UV-induced mutant of CS101B, CS101B Pat$^-$, was isolated. It was unable to grow on putrescine, arginine, and ornithine, but retained the ability to grow on GABA (see Table 2). The mutant exhibited 10% of the wild-type level of PAT activity, while retaining wild-type levels of the other enzymes of the pathway (Table 1). The mutant exhibited normal rates of putrescine, ornithine, and arginine transport. The Pat$^-$ mutant gave growth yields of 10% of the wild-type yield on limiting concentrations of arginine, ornithine, and putrescine while the yield on GABA was the same as in wild type. Back transduction of the mutant by a phage P1 lysate of the wild-type parent CS101B resulted in the return of the wild-type characteristics.

Table 3
EFFECT OF CATABOLITE REPRESSION AND NITROGEN AVAILABILITY ON ENZYME ACTIVITIES IN CS101B

	Enzyme activity (nmol/min/mg protein)				
Media[a]	**ADC**	**ODC**	**AUH**	**PAT**	**Prrd**
Glucose-NH_4^+	181	182	7	1	57
Succinate-NH_4^+	195	180	194	20	964
Glucose-glycine	177	171	210	36	1648

[a] Succinate (1%), or glucose (0.5%), served as carbon source; $(NH_4)_2SO_4$ (0.1%), or glycine (0.2%), served as nitrogen source.

In an interrupted-mating experiment with Hfr CS101B Met^+ as the donor, the *pat* gene was located close to *metB* at minute 89.[24] Transductional two-point crosses established a cotransduction frequency of 88% between *pat* and *metB*, 28% between *pat* and *rhaA*, and 0% between *pat* and *glnA*.

Analysis of three-point cross transductions between CS101B Pat^- and an *argH* strain AT2682 established the gene order *met pat arg*.

E. Isolation and Characterization of a CS101B Prr^- Mutant

In an attempt to determine whether in *E. coli* K-12 as in *P. fluorescens*[25] the product of the transamination of putrescine by PAT, γ-aminobutyraldehyde, is converted to GABA, a pyrrolidine nonutilizing mutant was isolated. γ-Aminobutyraldehyde exists in equilibrium with the cyclic compound Δ ′-pyrroline and pyrrolidine is the stable, commercially available reduced form of pyrroline. The mutant CS101B Prr^- grew on GABA as well as the wild-type parent, but its growth yield on arginine, ornithine, and putrescine as nitrogen sources was only 30% of that of the wild type. The mutant did not grow at all on pyrrolidine and exhibited about 10% of the PrrD activity of the parent strain, while showing wild-type levels of the other enzymes surveyed (Table 1). The *prr*$^-$ mutation was located on the *E. coli* K-12 chromosome[24] between 28 and 32 min by conjugation. Lack of mapped genes in this region made it impossible to carry out three-point crosses in order to establish the precise location of the *prr* gene.

III. REGULATION OF ENZYME SYNTHESIS IN THE ARGININE/ORNITHINE CATABOLIC PATHWAY

A. Catabolite Repression

Table 3 summarizes the effects of catabolite repression on the enzymes of the arginine, ornithine, and putrescine degradative pathways. The synthesis of AUH and PrrD is very strongly repressed by glucose in the presence of an abundant supply of nitrogen. The repression is relieved by substituting succinate for glucose (compare lines 1 and 2, Table 3) and is relieved even further by limiting the availability of nitrogen, substituting glycine for the ammonia (line 3, Table 3). In contrast, the synthesis of ADC and ODC is neither repressed by glucose, nor derepressed by limitation of the nitrogen supply.

B. Effect of cAMP

The repression by glucose of the synthesis of certain enzymes (catabolite-repression-sensitive enzymes, e.g., β-galactosidase) has been shown to be due to the low (cAMP) content of glucose-grown cultures. Addition of cAMP greatly reduces, or even abolishes catabolite repression.[26] Mutants defective in adenylate cyclase activity and therefore having very low internal concentrations of cAMP, are unable to grow on any one of several sugars, because the synthesis of the enzymes involved in their metabolism is dependent on cAMP,[27] which forms a complex with the catabolite gene activator protein (CAP), essential to promote mRNA synthesis.[28] Mutants with a lesion in the *crp* gene responsible for the synthesis of the CAP protein, do not respond to exogenously supplied cAMP, since the binding protein is not synthesized. In order to examine the mechanism of catabolite repression of the enzymes AUH, PAT, and PrrD, the *cya* mutation (the *cya* gene encodes adenylate cyclase, the enzyme responsible for the synthesis of cAMP) and the *crp* mutation were introduced into the wild-type strain CS101B. In addition, the *crp* mutation was introduced into the *cya* derivative of CS101B, thus resulting in a double mutant strain (Cya^-Crp^-). Table 4 summarizes data on the effect of cAMP on enzyme synthesis in the arginine, ornithine, putrescine, and GABA catabolic pathways. Data on the synthesis of β-galactosidase in the different cultures are given for comparison. Two different patterns may be distinguished. The synthesis of AUH responded to changes in the concentration of cAMP in the medium in a manner identical to that of β-galactosidase. Growth of the wild-type strain on glucose resulted in repression of AUH synthesis which could be fully relieved by the addition of 10-m*M* cAMP. In the wild-type strain grown on succinate with no cAMP added, AUH activity was high and increased only slightly when cAMP was added to the growth medium. The cya^- mutant grown in either glucose of succinate, in the absence of cAMP, showed severely repressed levels of AUH activity. Addition of cAMP to either medium fully relieved the repression. The Crp^- mutant and the Cya^-Crp^- double mutant showed very low AUH activity and did not respond to the addition of cAMP to the growth medium. The synthesis of PAT and PrrD was also severely repressed by glucose, but did not increase significantly upon addition of cAMP to the growth medium. In agreement with these findings and in contrast to AUH, the PAT and PrrD activities of the Cya^- and Crp^- mutants grown in succinate medium with no cAMP were high and did not increase significantly upon the addition of cAMP. Catabolite repression of GSST and SSDH, the enzymes of GABA catabolism, also exhibited a cAMP-independent component, as has been reported previously.[20]

C. Control by the *ntr* Regulatory System

That the escape synthesis of the enzymes of GABA catabolism upon nitrogen limitation depends on the *gln* regulatory system has been reported previously.[19] To see whether the enzymes AUH, PAT, and PrrD were also controlled by the *gln* regulatory system, a *gln* mutation that prevents derepression of GS synthesis and thus results in a glutamine requirement, was introduced by transduction into the wild-type CS101B (CS101BG). In parallel, a *glnL* mutation, which brings about constitutive derepressed synthesis of GS, was introduced into CS101B (CS101BC). The two strains were grown in glucose-ammonia minimal medium (conditions of catabolite repression and nitrogen excess) and in glucose-glutamine medium (conditions of catabolite repression and nitrogen limitation). As shown in Table 5, substitution of NH_4^+ with glutamine resulted in the release of AUH, PAT, PrrD, GSST, and SSDH from catabolite repression by glucose in strain CS101B. In strain CS101BG AUH, PAT and PrrD were not derepressed upon nitrogen limitation. In the constitutive mutant CS101BC the synthesis of AUH, PAT, and PrrD was derepressed regardless of the state of nitrogen availability. Data on the synthesis of GABA enzymes are included for comparison.

D. Induction

In *K. aerogenes* the synthesis of enzymes catalyzing the degradation of nitrogenous compounds such as arginine or agmatine is inducible.[4,5] In contrast, the GABA degradative pathway in *E. coli* K-12 is not an inducible system.[29] Since the degradation of arginine, ornithine, and putrescine in *E. coli* leads to the formation of GABA, it was of interest to

Table 4

EFFECT OF cAMP ON ENZYME SYNTHESIS IN WILD-TYPE, Cya$^-$, Crp$^-$, AND Cya$^-$ Crp$^-$ STRAINS

Strain[a]	Growth medium[b]	Enzyme activity[c]					
		β-galactosidase	AUH	PrrD	PAT	GSST	SSDH
CS101B	Glucose-NH_4^+	1.34	11	83	1	162	79
CS101B	Glucose-NH_4^+ + 10 m*M* cAMP	7.38	214	45	1	144	79
CS101By83	Glucose-NH_4^+	1.32	10	71	2	173	185
CS101By83	Glucose-NH_4^+ + 10 m*M* cAMP	7.94	185	24	1	166	197
CS101BCrp$^-$	Glucose-NH_4^+	1.31	10	120	2	154	268
CS101BCrp$^-$	Glucose-NH_4^+ + 10 m*M* cAMP	1.27	25	51	2	167	132
CS101By83Crp$^-$	Glucose-NH_4^+	1.29	15	80	2	173	161
CS101By83Crp$^-$	Glucose-NH_4^+ + 10 m*M* cAMP	1.37	24	46	2	144	232
CS101B	Succinate-NH_4^+	7.72	173	830	22	405	567
CS101B	Succinate-NH_4^+ + 10 m*M* cAMP	8.36	228	781	23	457	670
CS101By83	Succinate-NH_4^+	1.75	14	1036	20	401	572
CS101By83	Succinate-NH_4^+ + 10 m*M* cAMP	8.75	210	888	27	500	785
CS101BCrp$^-$	Succinate-NH_4^+	1.75	14	823	23	349	510
CS101BCrp$^-$	Succinate-NH_4^+ + 10 m*M* cAMP	1.40	12	919	25	359	558
CS101By83Crp$^-$	Succinate-NH_4^+	1.51	14	860	23	410	627
CS101By83Crp$^-$	Succinate-NH_4^+ + 10 m*M* cAMP	1.58	10	1001	27	339	734

[a] CS101By83 is a Cya$^-$ derivative of CS101B; CS101BCrp$^-$ is a Crp$^-$ derivative of CS101B; CS101By83Crp$^-$ is a Crp$^-$ derivative of CS101By83.

[b] Glucose (0.5%); succinate (1.0%); $(NH_4)_2SO_4$ (0.1%); IPTG added at 10^{-3} *M*.

[c] Enzyme activities are expressed as nmol/min/mg protein except for β-galactosidase which is expressed as OD_{420}:OD_{600}.

Table 5
ENZYME ACTIVITIES IN WILD-TYPE AND *gln* MUTANT STRAINS

		Enzyme activity[c]				
Strain[a]	**Media[b]**	**AUH**	**PrrD**	**PAT**	**GSST**	**SSDH**
CS101B	Glucose, NH_4^+	16	158	4	200	77
CS101B	Glucose, glutamine	238	1666	43	442	320
CS101BG	Glucose, NH_4^+	15	217	2	67	ND[e]
CS101BG	Glucose, glutamine	14	225	2	84	27
CS101BC	Glucose, NH_4^+[d]	228	1466	47	742	ND[e]
CS101BC	Glucose, glutamine	240	1433	49	522	442

[a] CS101BG is a *Gln*⁻ derivative of CS101B; CS101BC is a GlnC derivative of CS101B.

[b] Glucose (0.5%); $(NH_4)_2SO_4$ (0.1%); glutamine (0.1%).

[c] Enzyme activities are expressed as nmols/min/mg protein.

[d] L-glutamine, 25 μg/mℓ was added to CS101BG cultures.

[e] ND, not determined.

see whether this pathway is controlled by induction. In order to avoid the problem of the endogenous pool of arginine, the *argA*21 lesion of strain AT713, that blocks synthesis of arginine, was introduced by transduction into CS101B. The induction experiments were carried out with this arginine-requiring derivative of CS101B (CS101B713). Addition of L-arginine, agmatine, and L-ornithine to cultures growing in a glucose-glutamine medium at 30° C (condition of nitrogen limitation) did not enhance ADC or ODC synthesis. However, addition of L-arginine or agmatine but not L-ornithine, putrescine, or GABA, resulted in a three- to four-fold higher rate of AUH synthesis. The synthesis of PAT was only slightly enhanced in the presence of L-arginine, agmatine, L-ornithine, and putrescine in the medium.

In a set of experiments designed to determine whether PrrD is induced by putrescine and pyrrolidine as in *Pseudomonas*,[30] CS101B was grown in various media and the levels of PrrD were assayed. The results showed that there was no substrate induction of PrrD by putrescine or pyrrolidine.

The genes, which code for ADC, AUH, and ODC, *speA*, *speB*, and *speC*, respectively, are clustered at 64 min,[31] with *speA* and *speB* very closely linked to one another.[23] It was expected that these two genes would behave as one regulatory unit. However, our results showed that this was not the case. AUH responded to induction by L-arginine and agmatine, to repression by glucose, and to derepression by nitrogen limitation (Table 3). ADC and ODC synthesis did not respond to any of these controls (Table 3). These findings are in agreement with the obsevation of Hafner and Tabor[32] that bacteriophage insertion into either *speA* or *speB* did not affect the expression of the other gene, indicating that the two genes are not in the same operon.

The results showing a lack of response of ADC and ODC synthesis to different carbon sources for growth differ from the results of Boyle et al.[33-35] who found that the synthesis of ADC, ODC, and AUH in *E. coli* was actually enhanced in the presence of glucose as the carbon source. The positive effect of glucose, according to Boyle, is due to the low level of cAMP in glucose-grown cells. This is in direct contrast with our finding that glucose represses and cAMP depresses AUH synthesis (Table 4). It is possible that the discrepancy between our results and those of Boyle may be explained by differences between the strains used. Indeed, when we tested the *cya* mutant used by Boyle, strain CA8303, for its synthesis of ADC and ODC, the latter was repressed by the addition of cAMP, as reported by Boyle,[35] while in the *cya*⁻ derivatives of strain CS101B ADC and ODC synthesis was not affected by cAMP. Wright and Boyle report[35] growth rates of their strains on glucose and succinate that are much slower than those of CS101B and its derivatives. Our *cya* and *crp* derivatives of CS101B grew on succinate somewhat better than the strains used by Boyle (doubling time of 3.2 hr vs. 4.8 hr). This may be due to secondary mutations permitting better utilization of succinate by CS101B mutants. As seen in Table 4, the synthesis of β-galactosidase in the *cya* and *crp* derivatives of the wild-type parent CS101B responds in a classical fashion to repression by glucose and relief of this repression by the addition of cAMP. Another discrepancy between our findings and those of Boyle concerns the utilization of agmatine as a nitrogen source. Satischandran and Boyle[34] report that their strains cannot use agmatine as a nitrogen source, while our strain CS101B does utilize agmatine.

Canellakis and associates have recently shown that the activity of ADC and ODC in *E. coli* is inhibited by basic proteins (antizymes), the synthesis of which is induced by polyamines.[36] Since ADC and ODC each catalyzes the respective first step in pathways leading to the biosynthesis of polyamines, substances playing an important role in the regulation of the cell cycle, the control of these enzymes by polyamines makes good sense.[37,38]

In summary, the control of the arginine and ornithine degradative pathway in *E. coli* K-12 acts upon information on the supply of carbon and nitrogen and information on the level of polyamines, end products of this pathway, and effectors of the cell cycle. Fine tuning to the different signals is achieved by targeting them to different enzyme of the pathway. Thus, ADC and ODC seem to be exclusively controlled by polyamine levels,[36] AUH is the only element regulated by cAMP-CAP mediated catabolite repression, and PAT, PrrD, and the *gab* enzymes mainly respond to cAMP-independent catabolite repression. In each case, catabolite repression can also be circumvented by activation through the *gln* regulatory system in response to nitrogen limitation.

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Section B.
Polyamines and Plants

Chapter 7

POLYAMINES AND PLANT RESPONSE TO STRESS

Arthur W. Galston

TABLE OF CONTENTS

I. Introduction and Overview 100

II. Relation of Polyamine Metabolism to Physiochemical Stress 100
  - A. Osmotic and Water Stress 100
  - B. pH Stress 103
  - C. Nutritional Stress 103
  - D. Temperature Stress 104
  - E. Atmospheric Pollutants 104

III. Relation of Polyamine Metabolism to Infection of Higher Plants by Fungal Parasites 105

References 105

I. INTRODUCTION AND OVERVIEW

It has been known for the last 30 years that higher plants exposed to suboptimal or stress-inducing conditions of the environment may respond by accumulating high concentrations of the diamine putrescine (Put; 1,4-diaminobutane). The original observation on this subject, by Richards and Coleman,[1] dealt with barley plants grown in hydroponic culture and exposed to restricted levels of K^+ ions. Since then the following stresses have also been shown to result in the accumulation of Put: water deprivation,[2] high external osmotic concentration (best interpreted as water stress),[3,5] high concentration of NH_4^+,[6,7] H^+,[8] and other monovalent cations[9] in the ambient solution, exposure to the atmospheric pollutants SO_2[10] or Cd^{2+},[11] low temperature in subtropical crops,[12] and probably also near-UV radiation.[13]

The occurrence of a single biochemical response to such a diverse array of stress-inducing conditions suggests that Put accumulation may be taken as an indicator of stress metabolism in the plant. If this is true, then it could either represent the *cause* of the stress-induced injury syndrome or the metabolic *response* of the plant to the challenge from without. If it is the cause, we should expect to find that high Put is toxic to the organism; if it is a response, we should expect Put to alleviate or counteract injury-producing stimuli. The literature contains justification for both points of view[14,15] and this question is still not settled. It may of course also be that the accumulation of Put, although linked to stressful conditions, is neither a cause nor an effect of injury, but merely an incidental correlate of stress-induced metabolism, and therefore irrelevant to stress. This important question has still not been settled, although some approaches to it have been made.[16]

Put normally arises from the amino acid arginine through one of two major pathways: via ornithine and ornithine decarboxylase (ODC) or via arginine decarboxylase (ADC) and agmatine[17] (see Figure 1). Put is then usually converted to spermidine (Spd) and spermine (Spm) by successive additions of aminopropyl groups from *S*-adenosylmethionine (SAM), catalyzed by Spd synthase and Spm synthase, respectively. Put may also be diverted into other metabolic pathways, terminating in such products as γ-aminobutyric acid (GABA) pyrroline, various alkaloids, conjugates with various phenolic acids, and conjugates with proteins.[18] Clearly, the accumulation of Put could result from (1) increased synthesis of Put via ADC or ODC, (2) biosynthesis of Put by way of a new biochemical pathway not present in unstressed plants, or (3) decreased conversion of Put to Spd, Spm, alkaloid, GABA, or other metabolites. Our best current evidence indicates that Put accumulation, at least in cereals, is due mainly to *de novo* synthesis of ADC,[19] although there is also evidence of decreased Spd synthase activity.[20] The stress-induced accumulation of Put thus probably represents effects on transcription or translation in multiple, linked genetic systems. If this is indeed the mechanism, then the speed of the response (see below) is indeed remarkable.

It is known that plants exposed to drought also accumulate other nitrogenous materials, such as proline[21] and betaines.[22] This suggests that polyamine (PA) accumulation during stress may represent only the tip of the iceberg of nitrogen metabolism that is affected by stress conditions. It may well be that some master reaction, yet to be uncovered, accounts for the control of ADC, Spd synthase, and the synthesis of proline, betaines, and perhaps other compounds as well.

II. RELATION OF POLYAMINE METABOLISM TO PHYSIOCHEMICAL STRESS

A. Osmotic and Water Stress

Oat seedling leaves show a remarkably rapid response to osmotic stress.[2-5] If such leaves are excised, peeled to remove the lower epidermis and floated on 1-m*M* pH 5.8 phosphate buffer in the light, then the Put level remains constant at about 20 nmol/g fresh weight for

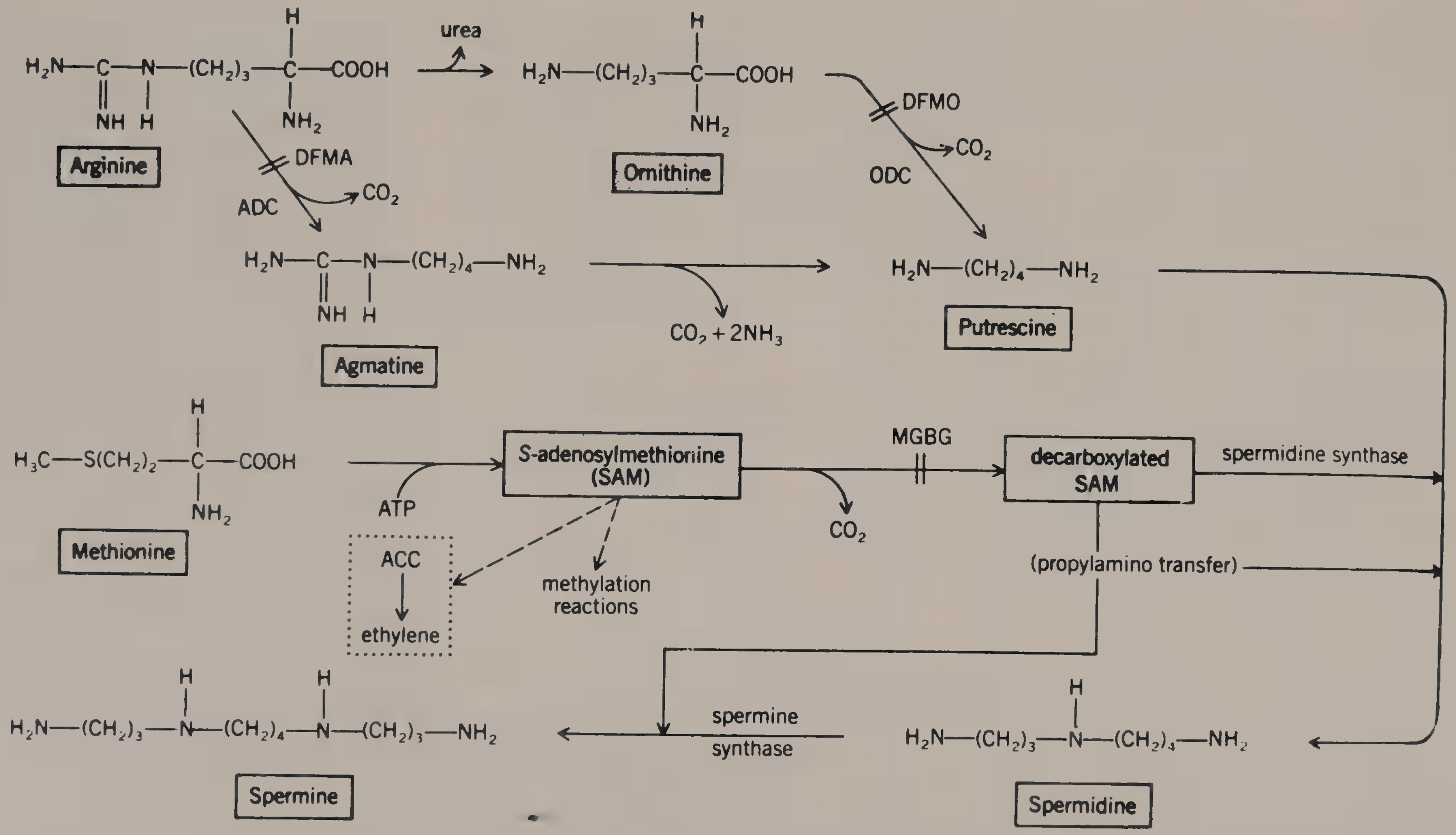

FIGURE 1. Pathways of polyamine biosynthesis in higher plants. ADC = arginine decarboxylase, ODC = ornithine decarboxylase, DFMA = α-difluoromethylarginine, DFMO = α-difluoromethylornithine, MGBG = methylglutaryl-bis (guanylhydrazone), and ACC = 1-aminocyclopropane-1-carboxylic acid.

at least 16 hr. However, if 0.4 *M* sorbitol is added to the bathing medium, the Put concentration begins to rise within 1 hr and increases 40 to 50-fold within 6 to 10 hr. This increase can be inhibited by cycloheximide (CH) added at "time zero", together with the sorbitol. If the CH addition is delayed 1 hr, then 60% of its ability to inhibit Put rise is lost; if the delay is 2 hr, CH does not inhibit at all. Timed additions of CH between zero time and 1 hr after addition of 0.4 *M* sorbitol have shown loss of inhibitability in as little as 15 to 20 min. This shows that the primary stress-induced metabolic reaction occurs very rapidly.

The accumulation of Put is also inhibited by the addition of α-difluoromethylarginine (DFMA), a "suicide" inhibitor of ADC. The analogous inhibitor of ODC, α-difluoromethylornithine (DFMO), produces no inhibition. Instead, it produces a paradoxical 15 to 20% enhancement of Put rise, attributed to a stimulation of ADC activity that has been shown in vitro. From such experiments, it seems clear that 0.4 *M* sorbitol leads to Put accumulation through ADC activation, at least in part. Direct measurement of ADC activity confirms this, since it doubles within 4 to 6 hr while ODC activity is unaffected. Neither Spd nor Spm rises appreciably within the first 6 hr after sorbitol addition. Spd does rise after 8 to 10 hr but Spm actually declines slightly over the entire test period.

A sorbitol concentration of 0.4 *M* produces optimal Put accumulation in these excised oat leaf sections after 4 hr; 0.1 *M* produces a slight effect, 0.2 *M* about half-maximal effect, and 0.6 *M* a slight supraoptimal depression to about 80% of the maximal value. Other solutes such as mannitol, proline, and betaine are equally effective, while sucrose, possibly because of its rapid penetration, is only about one fourth as active as sorbitol, and polyethylene glycol of various molecular weights is completely ineffective. The rapid response to sorbitol osmolarity is shown by many cereals, including oat, wild oat, barley, maize, and wheat. It is probably common to at least all cereals.

The rise in Put titer in response to stress is greater in light than in dark. The difference between dark and light can be completely annulled by the addition of 1 m*M* arginine and partially annulled by the addition of 1 m*M* ornithine. This implies substrate limitation of ADC activity, since ornithine can be converted to arginine through transcarbamylation. It is also known that light can affect ADC activity through phytochrome.[23]

These results can be reproduced, although on a slower time scale, by merely withholding water from an intact, potted oat plant until it has wilted.[2] After 4 days of water deprivation, with plants actively transpiring in an illuminated growth chamber, Put level and ADC had risen significantly. These changes begin only after the 2nd day, and are coincident with measurable signs of stress, including wilting and protein loss. These two responses, to high osmolarity and to water deprivation, are not quickly reversed by the removal of water stress, either in the intact oat plant or in the excised leaf segments.

How relevant are these phenomea for problems of drought in the field? One way to find out is to compare plants with different drought-resistance characteristics. When barley cultivars with differing susceptibility to drought are compared with respect to the size of the Put rise in response to 0.4 *M* sorbitol in excised, peeled leaf segments in the light, only small differences are observed. Excelsior (a drought-resistant variety), Proctor (a drought-susceptible variety), and Prior (intermediate)-excised leaf segments all showed a roughly fivefold rise mediated by ADC.[2] Thus, drought resistance or susceptibility does not appear to be easily correlated with this system.

In cereals, drought conditions and wilting are often associated with a rise in abscisic acid (ABA). Could the rise in ADC activity and the rise in Put be secondary and tertiary effects of a rise in ABA? The addition of 5 to 20 μg/mℓ ABA to the buffer bathing peeled oat leaf segments does, in fact, produce a slight rise in Put after 4 hr but this is only a doubling, as compared with a 20-fold increase produced by adding 0.4 *M* sorbitol. It would appear doubtful that the rises in ABA and Put are closely linked, if at all.[2]

B. pH Stress

The effect of high acidity on ADC was first discovered in microorganisms,[25] which possess both a constitutive, biosynthetic ADC, and an arginine-inducible, so-called biodegradative form of the enzyme. Transferring the organism from pH 7 to acid conditions (pH 4) caused an increase in ADC activity from roughly 0 to 270 $\mu\ell$ CO_2 evolved per milligram dry weight per hour. The new activity is presumably attributable to the biodegradative form of the enzyme. In barley seedlings, feeding 0.025 *N* HC1 or H_2SO_4 through the roots causes a twofold increase in the activity of ADC and *N*-carbamylputrescine amidohydrolase in the first leaf.[8] Excised *Cucumis* cotyledons respond similarly.[26]

In the excised, peeled oat leaf segment system,[24] Put levels are low and stable at pH 8, 7, and 6, but increase rapidly and proportionately as the pH is progressively decreased, at least down to pH 3.5.[27] This pH effect is virtually independent of the buffer system employed. The increase in Put is accompanied by a decrease in Spd, implying an acid-induced inhibition of Spd synthase. The rise in Put at pH 4.0 is detectable within 3 hr and peaks at about 9 hr. Upon transfer of the tissue back to pH 6.0, the accumulated Put disappears at roughly the rate of its initial appearance. The kinetics and extent of Put rise are paralleled by change in the specific activity of ADC. Finally, if uniformly labeled ^{14}C-arginine is fed to acid-stressed leaf segments, highly labeled Put appears within 2 hr. As the specific activity of the Put slowly declines, that of Spd rises, but to a much lesser extent.

As in water stress, the rise in Put caused by low pH is inhibited by CH and DFMA. The CH effect is readily reversible, but the DFMA effect is not. When the two stresses, i.e., high osmolarity and low pH, are applied together, there is an additive effect on both Put titer and ADC activity, implying action at a common locus.[24]

Using excised, peeled oat leaf segments floating on test media, Young[24] showed that the increase in ADC activity was probably due to increased *de novo* synthesis of the enzyme. Application of ^{35}S-methionine to both stressed and unstressed media resulted in its incorporation into presumptive ADC, migrating as a 39 k-dalton band on denaturing polyacrylamide gels. However, the specific activity of the "stressed" band was about 28% higher than that of the control band.

The physiological significance of the increased Put titer during acid stress is indicated by the close synchrony between PA levels and malate during the daily oscillation of acid levels during the Crassulacean acid metabolism cycle.[28]

C. Nutritional Stress

As mentioned in the Section I, K^+ starvation results in large increases in Put titer.[1] Deficiencies of Mg^{2+} and Ca^{2+} also induce a rise in Put, but these rises are smaller and take longer to develop.[1] Although S deficiency has also been reported to be effective,[29] this single report has not been substantiated, and in one instance, it has been negated. K and P interact in affecting Put titer, in that rises in Put due to K^+ deprivation are optimal only if P in the medium is more abundant than K^+. P deficiency alone induces a rise in the titer of agmatine, the decarboxylation product of arginine and precursor of Put.[30,31] K^+-deficiency-induced rises in Put have been demonstrated in many dicots[32,34] and monocots,[35,27] and may be ubiquitous in angiosperms. High Put in leaves has, in fact, been proposed as a means of diagnosing mineral deficiency in the field.[33,34]

Put increase following K^+ deprivation is accompanied by a rise in the activities of ADC and *N*-carbamylputrescine amidohydrolase.[36,37] The incorporation of label from ^{14}C-arginine and ^{14}C-ornithine into Put is enhanced in K^+-deficient plants on both monocots and dicots.[29,32,38] All of these effects are reversed within 48 hr after addition of K^+ to the medium.[39] Rb^+ and Na^+ can substitute for K^+ to some extent, while Li^+ is completely ineffective. There also appears to be some organ specificity, since Put accumulation in K^+-deficient barley occurs only in the aerial organs. It is not known whether roots are capable of demonstrating the effect.

Young[24] studied the effects of K^+ deficiency on PA metabolism in oat seedlings grown under controlled conditions in acid-washed quartz sand on diluted Hogland's medium. Plants grown had a low and constant Put titer and ADC activity. Within 6 days of K^+ with 6-mM K^+/deprivation, the Put titer was elevated 15-fold, and after 12 days it was 50-fold the value of the controls. The effective controlling range was between 0.06 and 6 m*M* K^+. Addition of K^+ to the medium caused rapid reversal of the Put rise; thus, 6 days after addition of 6 m*M* K^+ to plants with 6300 nmol Put per gram fresh weight, the Put had declined to 2800 nmol/g fresh weight while the continuously stressed plants reached 7600 nmol/g fresh weight. The activity of ADC parallels Put titer in all these cases. Soluble ODC activity is unaffected by K^+ deficiency, but pelletable ODC, presumably chromatin bound, shows some increase. Substitution of other monovalent cations for K^+ showed the following relative activity, measured as ability to lower ADC from the "zero-cation" level: $Rb^+ > K^+ > Li^+ > Na^+$.

The NH_4^+ ion represents a special case in the study of stress-related alterations in Put titer, in that NH_4^+ is both a monovalent cation, a source of H^+ ions and a source of N. It is known[6,7] that Put increases when soybean seedlings received NH_4^+ salts as their sole source of N, and the level of Put is related to the level of NH_4^+ fed. ^{14}C-agmatine in such plants is rapidly metabolized to *N*-carbamyl Put and Put, and the activities of ADC and agmatine iminohydrolase increase. However, it appears that uptake of NH_4^+ releases H^+ ions into the medium, thus creating an acid-stress condition, in which cation uptake becomes more difficult because of the competition from H^+ ions.

Salinity is also known to cause an increase in Put.[40] This appears to be an ion effect, rather than an osmotic effect, since adition of only 50 m*M* NaC1 to the nutrient medium results in a sharp rise in the titers of Put and cadaverine (1,5-diaminopentane),[41] and ^{14}C-arginine is rapidly metabolized to Put in cotton grown with 42 to 84 m*M* Na_2SO_4. Furthermore, feeding Put at 1 m*M* or above produces necrosis characteristic of salinity itself.[41]

It is clear tht the nutritional state of the plant is frequently reflected in its Put titer. This intriguing system needs to be further investigated by modern molecular biological techniques.

D. Temperature Stress

Since many organisms synthesize new proteins as a result of exposure to elevated temperatures, several investigators have inquired whether ADC, which responds so well to other stresses, might also represent a "heat shock protein". There have been no reports of success from such investigations.

However, it has recently been shown that in several semitropical fruits that are very intolerant of low temperatures, exposures to about 5°C results in elevations in Put titer that accompany development of tissue lesions.[12] This topic remains to be developed further.

E. Atmospheric Pollutants

Several years ago, it was shown that SO_2, a common aerial pollutant resulting from combustion of S-containing fossil fuels, causes an increase in free and bound Put in peas.[10] SO_2 absorption into cells results in the formation of acids, due to its conversion into HSO_3^- and SO_3^{2-} ions. Its effects may thus also be attributable to the release of H^+ ions. Once again, cation absorption from the external medium may be disadvantaged by the external H^+, and Put accumulation internally may provide an alternative source of cations.

Treatment with $CdC1_2$ results in an elevated level of Put in bean and oat leaf disks floated on test solutions.[42] As little as 0.01 m*M* Cd^{2+} produces some effect, and maximum effects are produced by 0.10 m*M*. This response is inhibited by DFMA, showing that ADC is the source of the extra Put, but the level of ADC activity in the leaves seems not to be affected by Cd^{2+} treatment. $CdC1_2$ fed to the roots of intact oat plants in culture also produces a rise in Put in the leaves, the optimum concentration of Cd^{2+} for producing this effect being 10 m*M* or higher.

III. RELATION OF POLYAMINE METABOLISM TO INFECTION OF HIGHER PLANTS BY FUNGAL PARASITES

It appears that at least some fungi lack ADC and produce the Put they need for higher PA biosynthesis exclusively through decarboxylation or ornithine by ODC.[43] If PAs are indeed required for growth, then it would appear that infection of higher plants by fungal pathogens might be prevented by blocking ODC activity. Since the plant has ADC as well as ODC, it should be able to make sufficient Put for its needs, even if ODC is completely inhibited. Thus, we first showed[44] that 0.5 m*M* DFMO supplied to cultures of phytopathogenic fungi on synthetic medium resulted in a marked inhibition of growth, and that such inhibition is reversible by 0.1 to 1.0 m*M* Put or Spd. Birecka et al.[45] confirmed this observation by similar experiments with *Helminthosporium maydis*. Subsequently, in our experiments with bean plants[46] DFMO sprays were effective in preventing infection by uredospores of *Uromyces phaseoli*, the bean rust fungus, as measured by local lesions on leaves. The effective DFMO concentrations (0.5 m*M*) produced no abnormal symptoms on the host plants, and were also able to protect unsprayed parts of the same or other leaves. While a satisfactory explanation for this systematic action is not yet available, it appears that this system has promise for field control of plant diseases caused by fungi.

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Chapter 8

POLYAMINES IN PLANT GROWTH AND DEVELOPMENT

Nello Bagni

TABLE OF CONTENTS

I. Introduction 108

II. Polyamines and Plant Growth 108
  A. Polyamine Pattern During Dormancy and Natural Release from Dormancy 108
  B. Polyamines and Cell Cycle 109
  C. Polyamine Effects on Cell Division 111
  D. Polyamines and Plant Tumor 112

III. Polyamines and Plant Differentiation 112

IV. Evidence for Polyamines as Plant Growth Substances 114
  A. Polyamine Uptake, Subcellular Distribution, and Translocation 114
  B. Basic Problems of Polyamine Research and Future Studies 116

Acknowledgments 117

References 118

I. INTRODUCTION

The absolute requirement of polyamines for growth was first established in bacteria such as *Haemophylus parainfluenzae*[1] and afterwards in polyamine-deficient mutants of *Escherichia coli*.[2] Mutants requiring polyamines for growth have been isolated also in primitive eukaryotes such as several ascomycetes.[3,4] As of today only one mutant partly deficient in enzymes for polyamine synthesis has been isolated in higher plants.[5]

Since there are at least two pathways leading to putrescine production, two alternative approaches have been developed in order to study the plants and physiological function of polyamines in higher plants. The first approach, used only for higher plants, utilizes explants cultivated in sterile culture of the tuber of *Helianthus tuberosus*, which is temporarily deficient in polyamines during dormancy. The second approach utilizes inhibitors that specifically interfere with the various steps in the polyamine biosynthetic pathway. The use of these two approaches, the study of the effect of polyamines in plant differentiation, and the evidence in favor of polyamine uptake and translocation led us to define polyamines as growth substances for higher plants.

II. POLYAMINES AND PLANT GROWTH

A. Polyamine Pattern During Dormancy and Natural Release from Dormancy

For the study of the effect of polyamines and in general of plant growth substances, *H. tuberosus* can be considered a unique plant. In fact this plant, which is propagated almost exclusively by vegetative reproduction due to the sterility of its inflorescence, provides genetically homogeneous material. Another advantage is that when the tuber enters into dormancy in late autumn, the homogeneous storage cells of the medullary parenchyma, which constitutes the major part of the tuber, stop in G_1 phase of the cell cycle. Dormant tubers contain a very small amount of different growth substances such as indol-3-yl-acetic acid (IAA), cytokinins and gibberellins, and a large amount of inhibitors such as abscisic acid (ABA).[6] Polyamines are also present in amounts too low to sustain growth. It was possible to demonstrate that polysomes, which represent only a small percentage of the ribosomes isolated from dormant tubers, are unable to synthesize proteins if exogenous polyamines are not provided. On the contrary, ribosomes from tissues activated for 18 hr with IAA synthesize proteins without additional polyamines.[7]

During tuber dormancy the amount of the main nitrogenous storage compounds, namely arginine and glutamine, increases, reaching the maximum level at mid February. These compounds are utilized when dormancy release approaches. The cells are in fact programmed to deplete their reserve substances and die. The considerable decrease in these substances in the second half of March corresponds to a sharp rise in free polyamines (putrescine, but particularly spermidine and spermine) that are metabolically related to arginine and also to glutamine via ornithine. In parallel, a decrease in ABA-like substances takes place.[8] Furthermore, polyamines bound to nucleic acids, particularly to rRNA, increase during the period of storage substance accumulation and decrease thereafter in connection with the increase in free polyamines. The approach of dormancy release is also accompanied by an increase in the molar ratio putrescine/tRNA and a poly(A) RNA containing fraction.[9] Tightly bound polyamines also change during dormancy. After hydrolysis of the TCA-insoluble fraction, the percentage of bound spermine and putrescine during dormancy is higher than that of the free ones, while the opposite is true for spermidine.[10] Ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) activities are low during dormancy. The general increase in free polyamines in the storage parenchyma prior to tuber sprouting[8] could be interpreted, as suggested earlier[11] for bean cotyledons during germination, as an indication that these organic cations will later be translocated to developing sprouts. We are now working in this direction (see Section IV.A).

Table 1
SOME FEATURES OF THE MEDULLARY CELLS OF *HELIANTHUS TUBEROSUS* TUBERS DURING DORMANCY AND NATURAL RELEASE FROM DORMANCY

Feature	Dormancy (November to March)	Dormancy release (April)
Cell cycle phase	G_1	G_1
Function	Storage	Depletion and death
Hormones	Low amount	Increase
ABA-like substances	Present	Decrease
Nitrogenous storage substances (arginine, glutamine, asparagine)	Initial accumulation followed by decrease	Decrease
Free polyamines	Low amount	Increase
rRNA-bound polyamines	Increase	Decrease
Tightly-bound polyamines	Decrease	Increase
ODC and ADC activities	Low	Low
Ribosomes	Stationary (few polysomes)	Decrease
Reactivity to activation	Rapid (nucleic acids: synthesis > degradation)	Slow (nucleic acids: synthesis < degradation)

B. Polyamines and Cell Cycle

Further indications that polyamines may be involved in or may control growth processes are obtained by studying the artificial break of dormancy. In fact, when slices of the parenchyma tissue of *H. tuberosus* are excised from a dormant tuber and put in in vitro culture without exogenous hormones, the cells do not divide. On the contrary, when they are exposed to an appropriate growth hormone such as IAA or 2,4-dichlorophenoxyacetic acid (2,4-D), a great number of cells change their program from storage parenchymatic to meristematic cells. The latter are induced to divide synchronously for two or three cycles.

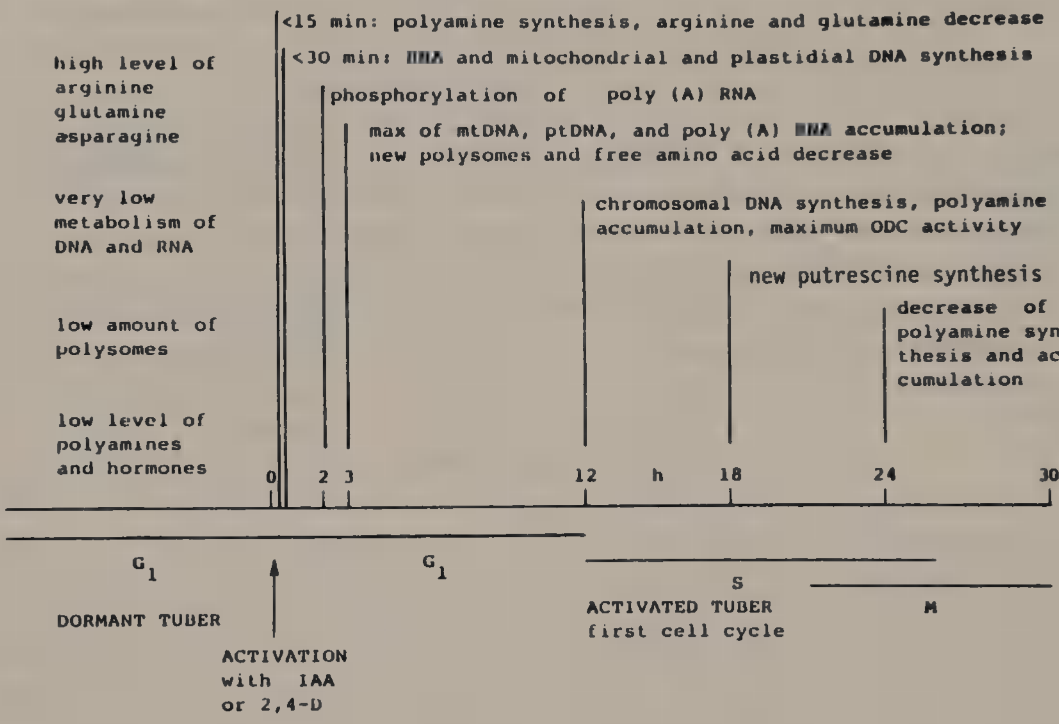
The different events that characterize dormancy and its artificial interruption are summarized in Tables 1 and 2. The study of the sudden switch of the cells from a slow metabolic activity, measured in months, to a very rapid one, measured in minutes and hours during the first cell cycle, sustained by an exogenous hormone such as 2,4-D or IAA allows us to demonstrate that the activation of polyamine biosynthesis is probably one of the first events occurring after the break of dormancy as it begins within 15 min from excision, whereas RNAs and nonchromosomal DNA synthesis probably begins slightly later.[12] The endogenous levels of free polyamines attained 1 hr after the break of dormancy are sufficient to stimulate protein synthesis in tuber slices. These levels are of the order of 10 to 100 μM depending on the polyamine, the same necessary to stimulate the growth of dormant tuber explants in vitro (see Section C).

The increase in polyamine synthesis and their accumulation, in particular that of putrescine, and the enhanced ODC activity in late G_1/early S phase is apparently a general phenomenon of cycling cells.[12-14] There is a temporal correlation between polyamine synthesis, ODC activity, and the start of chromosomal DNA replication. This may suggest that the late G_1 stimulation of polyamine synthesis is involved in the preparation for nuclear DNA synthesis and this was demonstrated with polyamine-deficient mutants in bacteria, yeasts, animals, and with inhibitors of polyamine synthesis. The pattern of ADC in *Helianthus* explants during the cell cycle is similar to that of ODC.[15,16]

In very early G_1-phase free arginine and glutamine decrease, new polysomes appear, and synthesis of mitochondrial and plastidial DNA takes place.[17,18] These events are necessary for the synthesis of new enzymes and for the supply energy to the redifferentiation cells. In addition, polyamines are known from in vitro studies to ensure that transcription and translation proceed correctly; therefore, their considerable rate of synthesis may be justified by

Table 2

PRINCIPAL PHYSIOLOGICAL FEATURES OF PARENCHYMA CELLS OF *HELIANTHUS TUBEROSUS* TUBERS DURING DORMANCY AND DURING THE FIRST CELL CYCLE AFTER THE BREAK OF DORMANCY


high level of arginine glutamine asparagine
very low metabolism of DNA and RNA
low amount of polysomes
low level of polyamines and hormones
<15 min: polyamine synthesis, arginine and glutamine decrease
<30 min: RNA and mitochondrial and plastidial DNA synthesis
phosphorylation of poly (A) RNA
max of mtDNA, ptDNA, and poly (A) RNA accumulation; new polysomes and free amino acid decrease
chromosomal DNA synthesis, polyamine accumulation, maximum ODC activity
new putrescine synthesis
decrease of polyamine synthesis and accumulation
0
2
3
12
h
18
24
30
G1
G1
S
M
DORMANT TUBER
ACTIVATION with IAA or 2,4-D
ACTIVATED TUBER first cell cycle

the requirements of the protein synthesis machine. Increasing amounts of polyamines, in particular putrescine, bound to the different nucleic acids were detected 3 hr after activation of tuber slices.[9] Tightly bound putrescine, spermidine, and spermine were also detected in the hydrolyzed TCA-insoluble fraction of slices excised from tubers during dormancy and at the beginning of dormancy release and activated for 3 hr. Tightly bound polyamines represented about 50% of total polyamines at the time of release from dormancy.[10] A second putrescine synthesis and polyamine accumulation begins during the progression of the S phase and reaches a maximum at the peak of the M phase followed by a decrease during the progression of mitosis.[12] This second increase in polyamine titer was also observed in several mammalian cell lines. Antimitotic agents, such as colchicine and vinblastine, also block ODC activity, whereas inactive structural analogues of these antimitotic agents do not. In addition, in hamster ovary cells, in HeLa, in 3T3, and in human fibroblasts PA2, treatment with methyglyoxal-bis-(guanyl) hydrazone (MGBG) and α-methylornithine induces an increase in the frequency of binucleate cells in addition to a decrease in spermidine. These binucleate cells exhibited a diffuse pattern of microfilaments compared with the control cells that show distinct actin cables as evidenced by indirect immunofluorescence using antiactin antibodies. These effects can be reversed by increasing the intracellular levels of polyamines.[19] Polyamines, therefore, seem to induce the polyamerization of G actin to F actin. In addition, polyamines can reverse the effects of cytochalasin B, a fungal metabolite that destroys the actin cables. Consequently, polyamines may have a role in the process of cytokinesis and cell division.

Another interesting model to study the relationship between polyamines and the cell cycle, developed by the groups of Mizrahi,[20,21] was the synchronous cell cycle of *Chlorella vulgaris*. In this organism, too, a peak in ODC activity precedes DNA synthesis, autospore release, and increase in cell number. No significant changes in ADC activity were observed. If ODC activity is essential for the synthesis of polyamines which in turn are essential for cell division, it is expected that inhibition of ODC activity would result in the inhibition of cell division. This premise was verified by studying the effect of α-difluoromethylornithine (DFMO) on the light-dependent enhancement of cell number and increase in polyamine content. The addition of DFMO inhibited cell division by 16.5 and 49% during the first and second cycle, respectively. This inhibition was partially reversed by putrescine. Exogenous putrescine caused only a small increase in cell number. Parallel to the inhibition of the increase in cell number, DFMO also inhibited the rise in putrescine and spermidine levels normally starting from the 8th hr of the cycle. In the second cycle the inhibition was more pronounced. The difference in the degree of inhibition of cell division between the first and second cycle was due to the presence of a substantial endogenous pool of polyamines, which reduces the inhibition at an early stage; in the following cycle, this pool has already been partially exhausted. Spermidine synthesis is inhibited in the second cycle. In regard to the different activities of ODC and ADC in cell function one possible explanation could be their different compartmentation in the cell as recent results on mithocondria of *Helianthus* tubers and chloroplasts of *Pinus radiata* cotyledons suggest.[22]

C. Polyamine Effects on Cell Division

Like other growth substances that are temporarily present in very low amounts in dormant tubers of *H. tuberosus* such as IAA, cytokinins, and gibberellins, polyamines, when supplied to the tuber explants, also stimulate RNA synthesis and amino acid incorporation and are able to sustain cell division.[7,23,24] The reactivity of the tissue to an artificial interruption of dormancy induced by polyamines or auxins varies according to the phase of dormancy and decreases when dormancy release begins. When spring approaches, the explants are capable of growing in absence of exogenous growth substances, since their endogenous content, particularly of polyamines, is now sufficient to sustain growth and is of the same order of magnitude as that required during deep dormancy to induce explant growth. The effect of exogenous polyamines in fact may not be seen in tissues which already contain a relatively large polyamine pool (i.e., generally all nondormant tissues). On the contrary, slices excised from dormant tubers of *H. tuberosus* put in culture without exogenous hormones do not divide, while the only presence of polyamines (spermine, spermidine, putrescine, and cadaverine), supplied at 10 to 100 μM in the medium, actively stimulates cell division. This growth stimulation is similar to that observed by IAA treatment.[23] However, in contrast to IAA, polyamines are inactive in elongation tests.

The effect of polyamines on cell division in higher plants, apart from *Helianthus* tuber that can be considered a unique plant for the characteristics previously described, has also been demonstrated in *Nicotiana sylvestris* cells in culture. A growth increment of 43% greater than in the control was observed at the optimal putrescine concentration of 90 μM. Putrescine at 50 to 100 μM also stimulated cell division in *Alnus glutinosa* and *A. incana* mesophyll protoplasts.[26] In the latter case the effect of exogenous putrescine was probably due to a lack or delay of polyamine synthesis in the protoplasts. The influence of polyamines on growth may be demonstrated not only by supplying them to polyamine-deficient tissues, but also by inhibition of their biosynthesis in growing tissues which may contain relevant amounts of polyamines. In plants, the study of polyamine inhibition is complicated by the fact that at least two pathways of putrescine synthesis are involved: one starting with arginine decarboxylation and the other which goes through ornithine decarboxylation. However, in some cases inhibitors of polyamine synthesis are used with considerable success in the elucidation of polyamine function. For example, in developing tomato ovaries after fertilization, ODC activity increased dramatically during cell division and later decreased to low levels during cell extension. On the contrary, ADC activity was not significantly different and always lower than ODC activity.[27] In this system 1 mM DFMO alone was able to completely inhibit ovary development by reducing ODC activity to a very low level, thus demonstrating the direct involvement of ODC in cell proliferation.[28]

In *Helianthus* tuber explants, short-term growth inhibition of cell cycle by MGBG and canavanine was difficult to obtain.[29] In the long term, however, canavanine and canaline, analogues of arginine and ornithine, respectively, completely inhibited explant growth. Good inhibition was also obtained with specific irreversible inhibitors such as DFMO, alone or in combination with α-difluoromethyl-arginine (DFMA).[30] However, other inhibitors of polyamine synthesis active in animals and bacteria do not affect explant growth,[6,14] probably due to a rapid modulation of ODC and ADC, as well as the induction of other enzymes such as citrulline decarboxylase found in *Helianthus* tuber, for which no specific inhibitor is available at present.[30] In any case, where inhibitors are used with success it appears that polyamine formation is necessary for growth and is not merely a consequence of growth. This evidence is particularly convincing when the inhibition is reversed upon adding polyamines.

D. Polyamines and Plant Tumor

Since polyamines appear to be among the important factors involved in growth and its regulation, the possiblity exists that polyamines might be connected with the abnormal growth of tumor cells. Higher amounts of putrescine and spermidine, but not of spermine, were always detected in all plant tumors examined to date: crown gall of *Scorzonera hispanica* induced by *Agrobacterium tumefaciens*, habituated tissue of *Scorzonera* and *Nicotiana glauca* induced by a hormone such as 2,4-D, and the genetic tumor *N. glauca* × *N. langsdorffii*.[31-33] In many tumors examined we found a positive correlation between increased levels of polyamines and of nucleic acids, especially at the beginning of the subculture. In particular in crown gall induced by the Ti plasmid of *Agrobacterium tumefaciens*, we found a significantly higher polyamine content and ODC activity in the crown gall than in the controls and a lack of arginase activity or the lack of its induction both in crown gall and *A. tumefaciens*, but present in the healthy control.[34] High polyamine titer seems to be a common feature of plant tumor tissues, as in some animal tumors, and seems to be independent of the etiological agent and positively related with the growth rate of the tissue. An attempt to correlate plant and animal tumors was made by Bagni and Serafini-Fracassini[32] using their own results on *Scorzonera* tumor and the data of Russell[35] on two rat hepatomas. It appears that in both organisms the fast-growing tumors (*Scorzonera* crown gall and rat hepatomas 3924 A) contain more putrescine and spermidine than the slow-growing tumors (*Scorzonera* habituated tissue and rat hepatoma 16) and much more than the controls. In summary, there is no apparent relationship between polyamines and tumor growth, but between polyamines and the growth rate of the tissue. No recent papers have been published on this interesting topic that could be further developed by studying the activities of those enzymes responsible for putrescine synthesis and their regulation; preliminary observations seem to suggest, in fact, a more considerable involvement of ODC than ADC.[34] In addition, the possibility of a transfer via the Ti plasmid of some genes involved in polyamine synthesis or degradation to plant cells could be an exciting field of investigation.

III. POLYAMINES AND PLANT DIFFERENTIATION

Polyamines as well as other plant growth substances such as auxins, cytokininins, or gibberellins, display a broad spectrum of action and are dependent on endogenous concentration, on their translocability, and on organ or tissue specificity.

While their action on cell division has been well documented, and in this respect polyamines resemble in particular cytokinins, the major difference between polyamines and auxins is that polyamines are not active in bioassays involving cell elongation. This in no way excludes the fact that endogenous polyamine content or the activity of the polyamine biosynthetic enzymes may change in response to cell elongation.[36] Many studies suggest that ODC is the key enzyme directly involved in cell division[28,37-38] while ADC is preferentially enhanced during cell enlargement, phytochrome-regulated growth, osmotic and acid stress, and potassium deficiency,[39,40] and its increase during cell proliferation, when it occurs, is only a consequence of growth. In recent years, several papers have been published which attempt to demonstrate a possible involvement of polyamines in cell differentiation more generally in morphogenesis. Interest in this topic was increased when people realized that polyamines could be rapidly synthesized from very common amino acids such as ornithine involved in urea cycle or arginine present in large amounts in plant cells. Few data are available on the effect of polyamines on cell differentiation. In *Helianthus* tuber explants grown in culture, it was observed that the differentiation of vascular bundles induced by polyamines at 100 μM was very similar to those induced by 0.35 μM IAA,[41] supporting the previously mentioned hypothesis that, like other phytohormones, polyamines displayed a broad spectra of action. Further direct evidence for polyamine action on morphogenesis is offered by the enhancement of flower bud differentiation in apple[42] and by the inhibition of regeneration in fragments of *Acetabularia* by α-methylornithine.[43] there is also a certain amount of other data supporting these observations: potato tuber sprouting is accompanied by a high endogenous content of polyamines and by an increase in ODC and ADC activities.

An area recently investigated by several research groups is root induction. In fact, nitrogenous compounds are among the endogenous factors which stimulate rooting of cuttings. Arginine, a putrescine precursor, seems to be particularly effective in stimulating root initiation in some species. Spermine exogenously supplied at 50-μM concentration, enhanced the number of adventitious roots developing on stem cuttings of *Phaseolus aureus* in the presence or absence of indolebutyric acid (IBA).[44] Spermidine did not affect or inhibit, at high concentration, root initiation. As expected, MGBG, a competitive inhibitor of *S*-adenosylmethionine decarboxylase, prevented the IBA-induced increase in spermine and markedly inhibited that in spermidine. As a consequence, root induction in cuttings treated with IBA and MGBG diminished with respect to the controls treated with IBA alone. These observations suggest that polyamines are involved in the early events in adventitious root formation. However, using the same plant material,[45] a stimulatory effect of exogenous polyamines on rooting was not observed. The *Datura innoxia* leaf explants growing *in vitro* are known to respond to different hormonal stimuli by giving rise to either callus, shoots, or roots. In this system ornithine and IAA displayed a synergistic effect on rizogenesis, while arginine was not effective.[46] Therefore, the possibility cannot be excluded that polyamines may be necessary for root induction since several inhibitors of polyamine biosynthesis block root formation while IBA treatment increases endogenous polyamine content. In addition, a high level of ODC activity in root tips declining towards older sections was observed in several plants such as potato, tomato, and corn, while ADC did not show a gradient in either tomato or potato.[47-49] Finally the localization of ODC as shown by binding with 5-^{14}C-DFMO, in the meristems of lateral roots of corn, formed during the 72-hr period following decapitation, suggested that polyamine content and biosynthesis may have a role in meristematic activity in corn roots.[48] Regarding adventitious shoot formation, some evidence that polyamines may be involved in this process was obtained with inhibitors of polyamine synthesis emptied in order to reduce or deplete endogenous polyamines.

Chicory root explants produce adventitious shoots in absence of exogenous growth regulators. In the presence of 2 or 5 mM MGBG or dicyclohexylamine (DCHA), the number of buds produced decreased significantly with respect to the controls. Fresh weight increments were also considerably reduced in the presence of the inhibitors; DFMO (up to 1 mM) did not exert any inhibitory effects either on fresh weight increase or on the number of buds formed.[50] On the other hand, in *Pinus radiata* cotyledons cultured in vitro, although spermidine titer was reduced to zero after a 5- to 10-day exposure to DCHA, adventitious shoot formation induced by benzylaminopurine (BAP) was not completely blocked by the drug.[51]

The contradictory data on the morphogenetic effects of polyamines could be due either to the difficulties arising from the interaction between the different hormones involved in this process, or to the lack of information concerning the ratio between free and bound polyamines. Some evidence as to how changes in this ratio can affect physiological processes has been gained only recently.

Another interesting model recently used to investigate the effect of polyamines on differentiation is carrot somatic embryogenesis. DFMA, at concentrations above 0.5 m*M*, significantly reduced or blocked embryo induction, but not DFMO except at 5-m*M* concentration which was regarded as toxic.[52] Putrescine, spermidine and spermine were equally effective in restoring embryogenesis in DFMA-treated cultures.[52] A similar inhibitor effect as that obtained with DFMA was obtained with canavanine, an analogue of arginine.[53] The spermidine synthesis inhibitors MGBG and DCHA were also found to reduce growth and embryogenesis in wild-carrot cultures.[54]

In view of these observations, Feirer and co-workers[54] concluded that putrescine and spermidine are among the essential factors required for embryogenesis. DFMA inhibited ADC activity by more than 88% and strongly reduced free polyamine content in the cells. DFMO, while inhibiting ODC activity, did not dramatically reduce putrescine or spermidine. The authors also noted that ADC activity was ten times higher than ODC activity, suggesting that the latter enzyme did not contribute significantly to putrescine biosynthesis. However, it must be pointed out that the authors do not indicate whether or not the pH at which they determined ODC activity (pH 7.1) was optimal under their conditions. Our experience shows that the optimal pH of ODC activity obtained from carrot cell is 8.3.[55] Even though ODC activity was high in these carrot cells, DFMO did not inhibit embryogenesis. The cells maintained the capacity to produce normal embryos and to differentiate them rapidly for as long as 21 days whereas only few embryos, blocked at the early stages, formed the canavanine-treated cultures.[53] In a similar study undertaken to examine the effect of DCHA on carrot embryogenesis, embryo formation was shown to be completely blocked in the presence of 5 m*M* DCHA.[56] Inhibition of embryogenesis could be reversed by simultaneously adding spermidine to the culture medium. Using a mutant cell line that grew at the same rate as wild-type cells but is selectively inhibited in its ability to undergo embryogenesis even under embryogenic conditions, these authors also showed that embryogenesis was specifically associated with increases in polyamine levels.

IV. EVIDENCE FOR POLYAMINES AS PLANT GROWTH SUBSTANCES

A. Polyamine Uptake, Subcellular Distribution, and Translocation

Up to a few years ago, evidence suggesting that polyamines could be considered plant hormones were very scarce.

Much of the evidence, such as their ubiquity, their high endogenous titer, their cationic nature, and fluctuation in response to the action of other plant hormones rather favored their role as "second messengers" in mediating intracellular hormonal effects.[7,39]

In addition, the lack of studies on their interactions with proteins and on their uptake and translocability could not disprove this hypothesis.

Our engagement in the possible demonstration that polyamines are plant growth hormones began almost accidentally. We noted that by spraying polyamines at concentrations ranging from 1 to 100 μ*M* on apples at the time of the fertilization processes, fruit set and yield per tree as well as fruit growth significantly increase, particularly when polyamines were applied during the cell-division stage.[42] In order to gain a deeper insight on the effects of polyamines during flower initiation and fruit growth, we then examined their uptake in short- and long-term experiments, their possible translocation, and the metabolism of exogenously supplied labeled putrescine in a single apple corymb. These results demonstrated the capacity of apple fruitlets, as well as young leaves, to absorb putrescine and to synthesize spermidine and spermine from putrescine. Putrescine and probably spermidine and spermine, were translocated via the peduncle from leaves to fruitlets and vice versa.

This transport does not seem to be polar. The presence of ^{14}C-label in the penduncle and at the bottom of the spur, may suggest that translocation via the sieve tubes had occurred without excluding a transport via parenchyma cells.[57] Since at the time no data were available on the mechanism of polyamine uptake in higher plants, this problem was further investigated by us in petals of *Saintpaulia ionantha* (African violet). The advantage of using this material was that the petal epidermis was thin, without stomata, cuticle, or waxes, thus favoring a rapid uptake. In other plant systems, leaves in particular, we observed that uptake was very slow due to the impermeability of the epidermis to hydrophilic compounds. The results using *Saintpaulia* petals showed that the uptake of labeled putrescine or spermidine or spermine was strongly dependent on the external pH and two maxima were observed both at low (0.5 μ*M*) or at higher external concentrations of polyamine (25 or 50 m*M*). One peak occurred at pH ranging between 4.5 to 5.5 depending on the polyamine and another peak at pH 8. The rate of polyamine uptake from the upper surface of the petal was constant at least for 1 hr and slowed down at 2 hr. The putrescine and spermidine absorbed were also metabolized to some extent (2% of the labeled polyamine absorbed was incorporated into spermidine and spermine, respectively) and spermidine was partly (6%) degraded to 1,3-diaminopropane.

Polyamine uptake over a 0.01 to 50- or 100-m*M* concentration range was linear at the lower external concentrations whereas at the higher concentrations, putrescine and spermine produced a typical saturation kinetics curve. The Km values for putrescine, spermidine, and spermine uptake were 8.6, 1.2, and 2.1 m*M*, respectively, with spermidine being the most absorbed at a low concentration (17 μ*M*). Polyamine uptake occurred against a concentration gradient at low external polyamine concentrations (0.5 to 100 μ*M*) and followed a concentration gradient at higher external concentration (100 μ*M* to 100 m*M*). Putrescine and spermidine did not seem to compete for the same site of absorption. Furthermore, putrescine and spermidine uptake was not inhibited by Ca^{++}, Mg^{++}, and K^{+} at the same concentration (17 μ*M*), where as 1.7 m*M* Ca^{++} inhibited and K^{+} enhanced spermidine uptake. Since polyamines are protonated at the pH values used in our experiments, we examined the interaction between the positive charges of the polyamine and the negative charges of the cell wall and membrane. By applying two different fractionation methods, one which is specific for the isolation of membranes and another for cell walls, we demonstrated that most of the putrescine (80 to 95%) was localized in the vacuoles and in the soluble fraction. A small amount of label was bound to membrane phospholipids and to the acidic groups of the cell walls. The method of petal infiltration with NaC1 confirmed that only 0.4 to 4.5% of the total putrescine absorbed was localized in the apoplast.[58-59]

To date, the methods applied to study the subcellular distribution of putrescine have not allowed us to distinguish between the soluble fraction and the vacuole. However, since the vacuole represents over 80% of *Saintpaulia* parenchyma cells, we are confident that the putrescine observed is localized in the vacuole. This does not mean that endogenous polyamines do not interact with other molecules and cell structures within the cells, as our data on subcellular localization in mitocondria and chloroplasts demonstrate, but that only a small percentage of exogenous polyamines enter them. In fact, Golberg and Perdrizet[60] found that in mung bean hypocotyl cells most of the endogenous putrescine was localized in the protoplasm and was not bound to cell walls, contrary to spermidine and spermine.

The driving forces for polyamine transport are not clearly identified. 2,4-Dinitrophenol (DNP) and carbonylcyanide-*m*-chloro-phenilhydrazone, two uncoupling factors, at various concentrations with or without Ca^{++} in the incubation medium did not affect polyamine uptake in *Saintpaulia* petals.[59] Nevertheless, in other plant systems such as carrot-cell culture in vitro,[55] 2,4-dinitrophenol inhibited putrescine uptake. At high concentrations of KC1 (above 17 m*M*), known to depolarize the membrane exerted an inhibitory effect on polyamine uptake in *Saintpaulia* petals, the negative internal membrane potential may be a driving force for spermidine uptake, while a pH gradient does not seem to be necessary.[59] Also, diethylstilbestrol, a proton-pump inhibitor, had no effect on spermidine uptake in *Saintpaulia*; however, at 0°C there was a 35% inhibition of spermidine uptake, compared with the controls kept at 20°C, as well as a 68% inhibition with 20 m*M* NaSCN. Thus, the polyamine uptake process in *Saintpaulia* petals is complex and may be dependent upon membrane potential, other unidentified factors, and only partially on an energy-dependent mechanism.[59] In order to better understand the mechanism of polyamine uptake, our studies have been extended to carrot cells cultured in vitro. In fact, this is a more active system which undergoes rapid cell division and is more responsive to exogenously supplied substances. Carrot cells display a very rapid uptake, reaching a maximum within 1 min after adding the labeled polyamine. The pH effect on uptake reflected the pattern previously found in *Saintpaulia* petals and increasing concentrations of exogenous polyamine produced a biphasic effect with different affinities at low- and high-polyamine concentrations. The interactions with Ca^{++} were also investigated and we found that this cation was not necessary for uptake to occur, but at 10-μ*M* concentrations it stimulated putrescine uptake and higher levels were markedly inhibitory. This stimulation was prevented by the presence of La^{++}, an inhibitor of calcium binding to the membrane and Ca^{++} uptake. Furthermore, 500-μ*M* La $(NO_3)_3$ inhibited putrescine uptake by 99.8%. We found a 34% inhibition also with 1-m*M* 2,4-DNP, but at the moment the mechanism of polyamine uptake has not been elucidated and research is in progress on this topic.[61] Since the intercellular and interorgan transport of a substance is one of the prerequisites for its hormonal function, this study is recently extended. Evidence shows that polyamines are taken up by plant tissues and are translocated over a considerable distance in xylem transpiration. ^3H-putrescine applied to intact roots of tomato seedlings at the cotyledon stage and of corn seedlings at the coleoptile stage moves rapidly to all the other plant organs; root pressure is sufficient to ensure polyamine translocation although xylem transpiration facilitates this movement.[62] In other plant systems such as *Nicotiana glauca* it was demonstrated that labeled cadaverine moved quickly from roots, where it is mainly synthesized via lysine decarboxylase to leaves, but not vice versa.[63]

B. Basic Problems of Polyamine Research and Future Studies

Many questions concerning the precise role of polyamines in cell growth remain as yet unanswered. For example, no specific protein receptors for polyamines have been found to date and the subject of polyamines bound to animal and plant proteins need to be further investigated. The topic concerning the covalent bindings of polyamines in plants is complicated by the fact that polyamines bind not only to proteins, but also to small acid-soluble molecules such as secondary metabolites (hydroxycinnamic acid, ferulic acid, coumaric acid, etc.).

In plant cells very little is known about protein-bound polyamines and derivatives. However, the binding of polyamines to low molecular-weight peptides in germinating apple pollen[64] and to proteins of 17.5 and 19 kdaltons, as well as some heavier ones, in activated slices of *Helianthus tuberosus* treated with ^{14}C-putrescine was demonstrated.[10] In animal cells, many of the covalent linkages between polyamines and proteins are catalyzed by Ca^{++}-dependent transglutaminases. The determination of this enzyme in plant cells could be useful for the understanding of the role of polyamines and to try to distinguish the covalent bindings from the other types of interactions with proteins. For example, it would be useful to isolate polyamine-binding proteins localized in the membranes, identified and partially purified in human platelets[65] in order to gain a better understanding of polyamine uptake and transport.

Another basic problem that must be solved concerns the significance of high intracellular levels of polyamines. In fact in normal plant cells undergoing active cell division, polyamine titers are approximately at m*M* levels reaching a maximum, in plant tumor cells, of 30 m*M*.

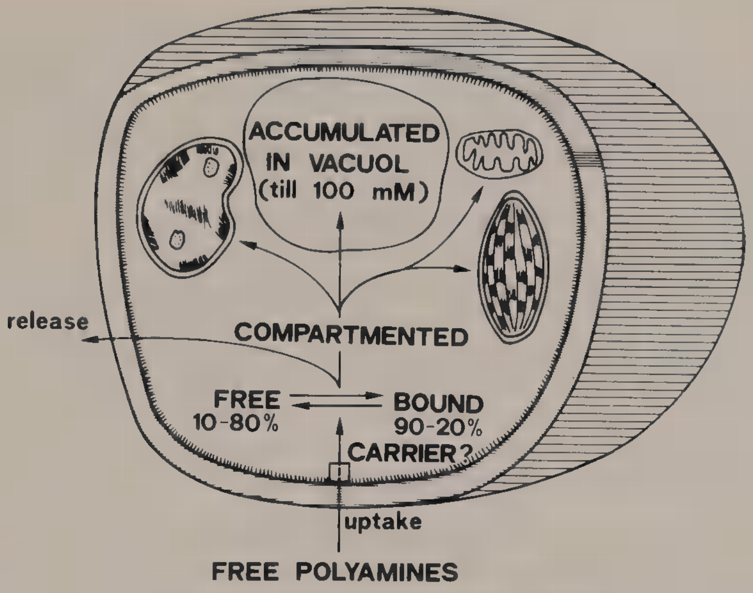


FIGURE 1. Schematic interpretation of polyamine movement, subcellular compartmentation, and changes in free and bound forms in a plant cell.

In these determinations, the polyamines conjugated to secondary metabolites whose physiological significance is still rather unknown were not taken into account. In particular cases polyamine levels, both free and bound to trichloroacetic acid-soluble peptides, are as high as 90 m*M* in apple pollen.[63]

On the other hand, as previously stated, the requirement for exogenous polyamines for cell division in the absence for endogenous polyamines or at levels lower than 1 μ*M* is about 10 to 100 μ*M*.

With the help of our present knowledge in animal biology we can attempt to find an explanation for these observations. It is well known, at least in animal systems, that the general mechanism by which Ca^{++} modulates a response is through a change in its concentration. These changes in Ca^{++} concentration represent a signal for enzyme reactivation, in particular for some protein kinases. Plant growth substances act by increasing free Ca^{++} concentration (μ*M* levels) in the cell. For this reason, polyamines which are also growth substances, could operate with an analogous mechanism. On the other hand, polyamines themselves are organic cations and in some cases they can substitute Ca^{++}. Certainly the mechanism of action of polyamines seems to be more complicated if we consider their specific effects which cannot be substituted by cations, for example, on the active conformation of tRNA and the previously cited bindings of polyamines to proteins. According to Slocum and Galston's hypothesis,[66] conjugated polyamines may be necessary to maintain free polyamines at more or less constant levels. However, this does not explain the high levels of free polyamines present in some cases, unless one assumes a subcellular compartmentation.[22,59] Figure 1 summarizes conclusive remarks on these fascinating molecules.

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Chapter 9

POLYAMINES AND PLANT HORMONES

Arie Altman

TABLE OF CONTENTS

I. Introduction ... 122

II. Plant Hormones ... 122
  A. Auxins ... 122
  B. Cytokinins ... 123
  C. Gibberellins ... 124
  D. Ethylene ... 124
  E. Abscisic Acid ... 125

III. Direct Interactions of Plant Hormones and Polyamines ... 125
  A. Auxins ... 125
  B. Cytokinins ... 127
  C. Gibberellins ... 128
  D. Ethylene ... 131
  E. Abscisic Acid ... 133

IV. Indirect, Putative Interactions with Plant Hormones ... 134
  A. Tissue and Cell Cultures ... 134
  B. Other Growth Effects ... 135
  C. Senescence and Stress ... 136

V. Comparative Functional Characteristics of Plant Hormones and Polyamines ... 136
  A. Plant vs. Animal Hormones ... 136
  B. Endogenous Concentrations and Exogenous Treatments ... 137
  C. Uptake and Translocation ... 137
  D. Molecular Aspects ... 138

VI. Concluding Remarks: A Bioregulatory Role for Polyamines? ... 139

Acknowledgments ... 140

References ... 140

I. INTRODUCTION

Polyamines which occur ubiquitously in plant tissues, have been recently shown to function in growth and differentiation processes in plants,[1-4] as well as in plant response to stress conditions.[5,6] Similar functions are mediated by known plant hormones (auxins, cytokinins, gibberellins, abscisic acid, ethylene, and other miscellaneous growth regulators) with more solid evidence as to their actual participation in growth control.[7-11] It is, thus, tempting to consider polyamines as another class of endogenous growth regulators of hormonal nature, albeit rigorous evidence is still lacking at this stage. The term "general bioregulators", however, seems to fit plant polyamines even better, as can be judged by recent numerous experimental data presented in this chapter. It is, therefore, of considerable interest to evaluate the different relevant properties of polyamines and their interactions with the known plant hormones. A concise survey of plant hormones will first be presented, followed by a review of the direct effects of hormones on endogenous polyamines and vice versa. Data concerning typical developmental processes which point to indirect, putative, interaction between polyamines and growth substances will then be discussed. Finally, several specific functional characteristics of polyamines and plant hormones, which are either similar or different, will be evaluated. The present review is selective and is limited to the more direct interactions of polyamines and plant hormones. Several aspects of this issue have been considered in recent reviews,[1-5] and in other chapters of this book.

II. PLANT HORMONES

The following is a concise survey of the known plant hormones and is intended for "polyaminers" which are unfamiliar with plant growth regulation. The five major plant hormones are auxins, cytokinins, gibberellins (GAs), ethylene, and abscisic acid (ABA) (Figure 1). As implied by their names, some of them refer in fact to a group of several substances which share similar functions and are closely related in chemical structure. Some are natural endogenous compounds while others are not present in plant tissues and are therefore collectively referred to as "growth regulators". In addition to these five groups of hormones, several other growth regulators are recognized, but have been omitted from the present survey, since they have seldom been investigated with respect to polyamines. Several functional aspects of plant hormones, as compared with animal hormones, will be discussed at the end of this chapter.

A. Auxins

The major endogenous auxin, and the first plant hormone that was discovered, is indole-3-acetic acid (IAA).[7] Other naturally occurring auxins, e.g., phenylacetic acid, are known. Synthetic compounds with auxin activity, sometimes more potent that IAA itself, include (1) substitution of the aliphatic side chain of the indole ring, e.g., indole-3-butyric acid; (2) a naphthalene aromatic ring, e.g., naphthalene acetic acid; and (3) halogenated phenoxy compound, e.g., 2,4-dichlorophenoxy-acetic acid (2,4-D). Tryptophan is the major precursor for IAA biosynthesis: transamination of tryptophan yields indole-3-pyruvic acid, which is then converted to indole-3-acetaldehyde and finally to IAA. Other pathways exist as well. Metabolism of IAA involves peroxidative decarboxylation, and oxidation to oxindole-3-acetic acid. In addition, IAA forms biologically active endogenous conjugates which are esters of the β 1 → 4 glucan type, or conjugates with inositol compounds. Physiological responses to exogenous auxins are manifold, and include effects at the cellular level (e.g., stimulation of cell division and expansion, shoot growth, xylem and phloem differentiation, root initiation in cuttings, and regulation of tissue morphogenesis in culture), as well as effects at the organ and whole plant level (e.g., seedling morphology with respect to light,

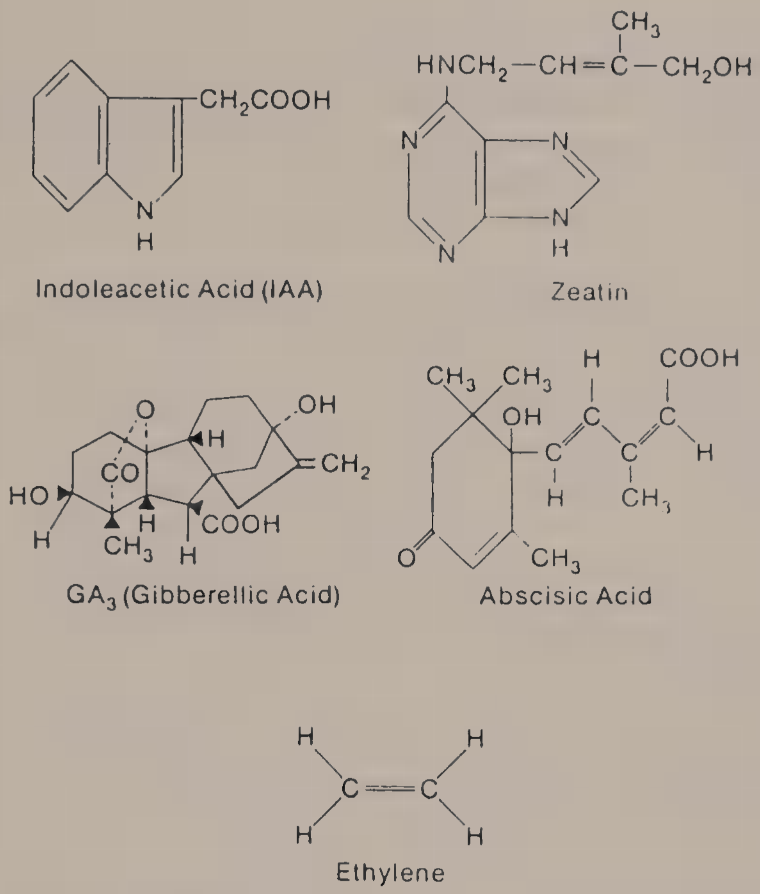

FIGURE 1. The five major plant hormones: auxins, cytokinins, gibberellins, abscisic acid, and ethylene.

apical dominance, abscission, fruit setting, and ripening, etc.). The suggested mechanism of IAA action involves attachment to a specific binding site, and initiation by means of a second messenger, of a cascade of reactions of two types: short-term membrane changes leading to media acidification and long-term changes in nucleic acids and proteins leading to enzyme synthesis or activation. A concomitant of these reactions is probably a change in cell wall plasticity which facilitates further growth.

B. Cytokinins

Cytokinin is the generic name for substituted purine compounds which promote cytokinesis in plant cells (amd more specifically, which, in the presence of auxins, induce cell division in plant tissue cultures).[8] All the known naturally occurring cytokinins are substituted 6-aminopurine (alternatively written as N^6-substituted adenine). Free cytokinins of plant sources include zeatin and related compounds such as zeatin riboside, dihydrozeatin, and others. Cytokinins are present also as constituents of transfer (t)RNA, e.g., N^6 (isopentenyl) adenosine. Kinetin (or 6-[furfurylamino] purine) and 6-(benzylamino) purine are two potent synthetic cytokinins. Cytokinin biosynthesis may involve two routes: (1) biosynthesis of free cytokinins (one step of which is a reaction between 5′-AMP and isopentenylpyrophosphate, catalyzed by isopentenylpyrophosphate:AMP-isopentenyltransferase, yielding i^6A-5′-monophosphate) and (2) formation of tRNA cytokinins, by the enzymatically catalyzed attachment of an isopentenyl side chain to adenine residues in tRNA. Applied cytokinins are rapidly metabolized by plant tissues, by two general routes: (1) modifications of the N^6 side chain (such as hydroxylation, saturation, and sugar and amino acid conjugation) in which the essential features and activity of the molecule are retained and (2) side-chain cleavage (involving possibly ‘‘cytokinin oxidase’’) which yields biologically inactive compounds such as adenine and adenosine. In addition to their effect on cell division, externally applied cytokinins (and in a few cases endogenous cytokinins) affect several physiological processes. These include, among others: morphogenesis (root-shoot formation) in tissue cultures, crown gall formation by *Agrobacterium* plasmids, leaf growth and shape by cell enlargement, delay of leaf senescence, bud growth, and substitution of light requirements in seed germination, pigment synthesis, and chloroplast development

C. Gibberellins

To date, 62 individual GAs are known, and of these, 51 have been isolated from higher plants.[9] All GAs have the same basic 20-carbon ring structure of *ent*-gibberellane. GA_3 (gibberellic acid) and a mixture of GA_4 + Ga_7 are the most widely used GAs for external application. GA biosynthesis involves formation of geranylgeranylpyrophosphate from mevalonic acid, and its cyclization which yield, among others, *ent*-kaurene and formation of *ent*-7a-hydroxykaurenoic acid. Several additional oxidative steps lead to the formation of GA_{12}-7-aldehyde which is the first intermediate with the GA ring structure and is probably common to all plants. Further metabolism involves methylations, hydroxylations, and oxygenations, and yields both bioactive and nonactive GAs. GA action has been primarily deduced from plant responses following treatments with GAs, especially GA_3, which result, in many plants, in promotion of shoot elongation and flowering. GAs can also promote plant growth by affecting either cell division or cell expansion, or both, probably by mediating auxin levels. It has been suggested that GA effect on cell expansion involves biophysical parameters such as cell osmotic potential and cell wall extensibility. In addition, GAs seem to be active in dormancy breaking and germination of seeds, in pigment biosynthesis, and in a variety of key metabolic reactions. This pattern, as well as molecular aspects of GA functions, have been studied primarily with respect to seed germination. Thus, GA_3 can substitute for the embryo of cereal seeds in inducing α-amylase activity in the aleurone layer, resulting in hydrolysis of the starchy endosperm reserves of the seeds. This is one of the best-studied molecular effects of plant hormones, and involves the promotive effect of GA on transcription of specific genes, which are then translated to a variety of hydrolytic (and other) enzymes.

D. Ethylene

Chemically, ethylene (C_2H_4) is the most simple and the only gaseous plant hormone.[10] Ethylene effects are elicited by applying either gaseous ethylene or 2-chloroethyl-phosphonic acid (ethrel), an ethylene-releasing compound. Ethylene is produced at various rates by essentially all parts of higher plants, reaching a production rate of a maximum recorded value of $3.4\ \mu\ell \cdot g^{-1} \cdot hr^{-1}$. Ethylene is ultimately derived from carbons 3 and 4 of methionine, and the biosynthetic pathway involves decarboxylation and cleavage of *S*-adenosylmethionine (SAM) which yields a stable compound, 1-aminocyclopropane-1-carboxylic acid (ACC), a reaction catalyzed by ACC synthase. ACC is the immediate precursor of ethylene, and its conversion to ethylene is oxygen dependent. The CH_3S moiety of SAM is released as methylthioadenosine (MTA), which is then converted to methylthioriboside (MTR) and the CH_3S is recycled into methionine. The pathway of ethylene biosynthesis may be, thus, coupled with polyamine biosynthesis, and will be discussed later. Ethylene biosynthesis is induced by auxins, and by wounding and stress conditions (‘‘stress ethylene’’), and is considerably enhanced during fruit ripening. Ethylene can be metabolized to ethylene oxide and CO_2, or converted to soluble metabolites such as ethylene glycol and its glucose conjugate. Ethylene influences all phases of plant development and all tissues, hence it has a broad regulatory activity. This includes spectacular effects such as enhancement of fruit softening and pigment changes, as well as other aspects of ripening. Leaf and flower abscission, leaf senescence, as well as effects on breaking of bud and seed dormancy, seedling growth, flower initiation, modification of sex expression, and several other processes are also affected by ethylene. Ethylene effects can be prevented or delayed by ethylene biosynthesis inhibitors such as aminoethyoxyvinylglycine (AVG) and aminooxyacetic acid (AOA) which inhibit ACC synthase, or by antagonists of ethylene action like CO_2 and silver ions.

E. Abscisic Acid

Of the few known endogenous plant inhibitors, abscisic acid (ABA) is the most widely distributed and the best characterized.[11] The natural enantiomer *cis* (+) ABA is one of the most intensely optically active compounds. Chemically synthesized ABA is racemic and composed of equal amounts of the mirror-image forms of (+) and (−) ABA enantiomers. In many reactions the (−)ABA is as active as (+)ABA, but it has been suggested that this form inhibits only the slow ABA reactions, whereas the (+) form inhibits both fast and slow hormonal reactions. ABA biosynthesis occurs by two main routes: (1) photooxidative cleavage of carotenoids such as violaxanthin, giving rise to the inhibitor 2-cis-xanthoxin which is rapidly metabolized to ABA and (2) direct biosynthesis from mevalonic acid in the terpenoid pathway. The latter is considered as the predominant mechanism of ABA biosynthesis. Inactivation of ''free'' ABA involves three mechanisms: (1) oxidation of ABA to form phaseic acid, which is then reduced to dihydrophaseic acid; (2) isomerization of ABA by light, resulting in a 1:1 mixture of *cis*-ABA and its 2-*trans* isomer which is biologically inactive; and (3) conjugation by formation of covalent links, with glucose or other monosaccharides; *trans*-ABA is conjugated with glucose and, is thus removed (when in combination with the active cis-ABA). ABA has been found in all higher plant tissues examined, and is usually a potent growth inhibitor, although in a few cases it may promote growth. It is usually accepted that ABA stimulated leaf abscission (although these tissue respond more sensitively to ethylene), and is involved in the induction and maintenance of dormancy of buds and seeds. The role of ABA in seed dormancy is better understood than in buds. Thus, ABA effects are opposed to those of GA in several systems, including the molecular regulation of α-amylase. Notable are cases of ABA involvement in several stress responses: chilling, salinity, and especially, leaf-water stress. The latter is accompanied by a large increase in ABA content and stomata closure, and it has been proposed that turgor pressure is the critical factor. Exogenous application of ABA may temporarily protect plants from desiccation, chilling injury, and other stress phenomena.

III. DIRECT INTERACTIONS OF PLANT HORMONES AND POLYAMINES

Experimental evidence for direct interaction between the five major plant hormones and polyamines are presented in the following. Most of these studies deal with the effect of applied hormones and related growth regulators on changes in polyamine content, activity of polyamine biosynthesis enzymes, and incorporation of precursors into polyamines. Only a few studies deal with the effect of applied polyamines and related chemicals on changes in endogenous plant hormones. Indirect putative interactions between polyamines and plant hormones, as may be concluded from typical growth responses, are discussed separately in Section IV.

A. Auxins

Auxins are the most studied group of plant hormones, and their effect on plant growth and development is manifold. Auxins are intimately involved in the control of cell division and extension growth, in a variety of differentiation processes such as embryogenesis and organogenesis, as well as in metabolic regulation of several physiological events. Thus, it is not surprising that the relevance of polyamines to plant growth was first studied in auxin-treated tissues.

Earlier work indicated that polyamines stimulate the growth of Jerusalem artichoke (*Helianthus tuberosus*) tissue explants in vitro similar to the stimulation by auxins.[12,13] This was eventually supported by observations that the enhanced growth rate of both habituated callus of *Nicotiana glauca* and of crown gall tissue of *Scorzonera hispanica*, which are both characteristic of auxin-stimulated growth, is accompanied by elevated levels of certain polyamines and increased putrescine (Put) biosynthesis.[14,15] Direct evidence, however, for the effect of auxins on polyamine content and metabolism was first provided in a series of studies with dormant vs. auxin-activated *H. tuberosus* tuber slices. Incubation of dormant tuber parenchyma explants in IAA resulted in a marked increase of spermidine (Spd) and spermine (Spm) content of both whole cells and the ribosomal fraction.[16] A subsequent, more elaborate study[17] clearly showed that activation (i.e., dormancy break) of tuber slices by 2,4-D brought about a rapid three- to ten fold increase in the contents of Put, Spd, and Spm. This increase was already evident 15 min after activation in the very early G_1 phase, and continued for at least 1 hr. Activation of tuber slices resulted also in a rapid, considerable decrease in glutamine content and less so in arginine (both possible precursors of polyamines). Similar changes, over longer periods of time, were also observed during the natural dormancy break until sprouting. Changes in free polyamines and related compounds were also followed during the entire 24- to 30-hr period following activation of slices by 2,4-D, corresponding to the first cell cycle.[18] A marked decrease in stored arginine and glutamine, and a corresponding increase in Put, Spd, and Spm content, were observed immediately after the dormancy break during the early G_1 phase concomitant to RNA synthesis. A second phase of polyamine accumulation, especially of Spd, occurred during the progression of the S phase (18 to 24 hr after activation), and was followed by a moderate decrease. Likewise, incorporation of 5-^{3}H arginine into polyamines also increased early during the first cell cycle, and decreased only towards its termination, but no significant changes in the activity of arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) of activated slices were found. The possible function of polyamine during the activation of a cell division in *Helianthus* tissue was also investigated with respect to binding to nucleic acids.[19] Bound polyamines were found in all the isolated RNA fractions (rRNA, tRNA, and a fraction which contains poly(A)RNA and proteins). The number of moles of bound polyamines was inversely proportional to their molecular weight. The amount of polyamines especially of Put bound to all three fractions, increased considerably during the first 3 hr after activation by 2,4-D. The relevance of polyamines to cell division in this system is further supported by studies with several analogues and inhibitors of polyamine biosynthesis.[20,21] Thus, canavanine and canaline, analogues of arginine and ornithine, respectively, inhibited the 2,4-D-stimulated growth and, to varying degrees, accumulation and synthesis of polyamines and the activities of ADC and ODC. MGBG showed no growth inhibition while inhibiting Spd and Spm synthesis at varying degrees, and partial growth inhibition was observed with millimole concentrations of DFMO and DFMA. Exposure of dormant *Helianthus* tuber explants to another auxin, naphthaleneacetic acid,[22] also activated cell division, and brought about rapid depletion of endogenous arginine and marked accumulation of polyamines, especially of Spd. No effect on ADC or ODC activity was detected, and labeled arginine and ornithine were not incorporated into Spd. The above-mentioned studies clearly show that activation of cell division by auxins in cultured *H. tubersus* explants is accompanied, and probably preceded, by considerable accumulation and synthesis of polyamines.

The interaction between auxins and polyamines was studied in yet another plant system which responded readily to auxin treatments, i.e., root formation in cuttings. Indolebutyric acid (IBA)-induced root formation in mung bean hypocotyle cuttings was accompanied by a considerable increase in Put and Spd levels, whereas senescing (unrooted) cuttings evinced a rapid decline in polyamine content.[23] These changes in polyamines were dependent on the IBA concentration and were organ specific, i.e., in hypocotyls and epicotyls but not in leaves. Exogenously applied polyamines and their precursors did not promote root formation.

Rooting was inhibited by canavanine and canaline, and this inhibition was reversed by arginine and ornithine, respectively, and MGBG also inhibited root formation. In a subsequent study,[24] it was found that the incorporation of labeled arginine and ornithine into Put and Spd is closely related to the pattern of root formation in the hypocotyl, i.e., a first peak of Put biosynthesis at 0 to 24 hr, corresponding to the period of root primordia formation, and a second peak of 48 to 72 hr, corresponding to root growth and elongation. Put biosynthesis was enhanced considerably by IBA in both phases. These results are also supported by a study with mung bean cuttings,[25] in which IBA was found to increase Put, Spd, and Spm levels prior to any development of root primordia. MGBG inhibited root formation and growth, in the absence or presence of auxin, and reduced the level of Spd and Spm while increasing Put content. In this study, however, Spm enhanced both the formation and elongation of adventitious roots by itself, as well as in the presence of IBA. These studies support the hypothesis that auxin-induced root formation may require the enhancement of polyamine accumulation and biosynthesis.

In addition, interrelationships between polyamines and auxins were studied in several other systems, and are discussed elsewhere in this chapter. Thus, naphthoxyacetic acid, which mimics natural pollination in tomato flowers, induced fruit setting concomitant with a 3-fold increase in ODC activity (as compared with a 4.7-fold increase in pollinated ovaries).[26] This is in agreement with previous studies[27] which showed that the basal ODC activity in ovaries of flowers at full anthesis increased tenfold 4 days after pollination. IAA, at 10^{-3} M induced a threefold increase in the activity of ODC during germination of barley seeds,[28] and the same auxin markedly increased ADC activity in rice embryos.[29] Auxin-induced ethylene production was inhibited by polyamine treatments in both Pinto leaf disks,[30] and in etiolated soybean hypocotyls.[31] Some inhibition of ADC activity in IAA-treated etiolated pea seedlings was also noted.[32] Finally, it is of interest to note that a putative specific role for arginine with respect to auxin-induced growth of *Avena* coleoptiles was already suggested in 1949.[33] In this study, arginine was found to be very effective in increasing the *Avena* growth response to IAA, and was inhibited by canavanine, the arginine analogue. In view of more recent studies, these results suggest that the specific effect of arginine, a precursor of Put, may be mediated via polyamines.

The close interaction of auxins and polyamines has been demonstrated satisfactorily in two plant systems: growth of *Helianthus tuberosus* tissue in vitro, and root formation in mung bean cuttings. Changes in polyamines during postpollination events and fruit set may also be related to an auxin effect. In some cases, changes in polyamines are evident before cell division or any other morphological changes in auxin-treated tissues, although, as yet, there is no substantial evidence that polyamines act as a second messenger of auxins. In view of the fact that IAA can cause so many different effects in different plants and tissues, it is especially important to elucidate the nature of its interaction with polyamines, which also changes dynamically during growth and development.

B. Cytokinins

Cytokinins, among other plant hormones, have a central role in the control of cell division and meristematic activity. In view of the suggested participation of polyamines in plant growth and development, it is however unfortunate that relatively little is known on the interaction of cytokinins and polyamines.

Short-term organ culture of cucumber cotyledons served to study the effect of cytokinins (and other plant hormones) on ADC activity and polyamine content.[34-36] Benzyladenine and its riboside benzyladenosine, and to a lesser extent kinetin, induced a marked increase in ADC-specific activity and in Put content of the expanding cotyledons. The same treatments, however, decreased Spd and Spm content. Exposure of cotyledons to KCl promoted growth and inhibited ADC activity and this inhibition was partially reversed by benzyladenine and kinetin. Cytokinins (as well as GA_3) were able also to reverse the ABA-induced inhibition of ADC activity. The stimulatory effect of cytokinins on ADC activity was abolished by cycloheximide, indicating *de novo* synthesis of the enzyme. In a subsequent work it was found that pretreatment of cotyledons with benzyladenine further increased, in an additive manner, the homoarginine-stimulated activity of ADC.[36] In a similar study,[37] several cytokinins increased Put content (while somewhat decreasing Spd, and especially Spm content) of barley seed endosperm. It was also noticed in this study that addition of kinetin to K^+ and Zn^{++} did not abolish the decrease of Put by these ions, but considerably increased Spm content. Kinetin and benzyladenine also increased (about twofold) Put content in light-grown lettuce cotyledons, evincing in addition some interaction with the effect of high concentrations of inorganic cations.[38] In a study with inbibed rice seeds, where changes in seed vigor and viability are correlated with polyamine content and metabolism, kinetin seemed to exert a considerable effect.[39] Treatment of high- and low-vigor seeds with 10^{-4} M kinetin resulted in a three- to four-fold increase in total polyamine content, the increase in Spd being the most dramatic. The activity of ADC was also enhanced by kinetin, though to a lesser degree, but the reverse was observed for the activities of diamine and polyamine oxidase. In another study,[29] ADC of viable rice embryos increased dramatically (about eightfold) on incubation with 10^{-8} M kinetin (and other plant hormones).

Interactions between cytokinins and polyamines were also noticed in other studies. Kinetin enhanced betacyanine efflux from disks of beet root, and Spm completely inhibited the kinetin effect,[40] which suggests to the authors that there is a common binding site on the membrane for polyamines and kinetin. In another study,[32] benzyladenine markedly increased the ADC activity in excised terminal buds of etiolated peas following exposure to red light. Benzyladenine was also effective in increasing concentrations of both Put and Spd in aged potato tuber disks.[41]

Several cytokinins definitely increase polyamine content and affect certain related enzymes. Cytokinine interaction with polyamines seems to be manifested in systems where the cytokinins are involved in augmenting growth or delaying senescence, but the available evidence is rather fragmentary and does not permit any solid conclusions to be made.

C. Gibberellins

GAs control several specific growth processes in plants and are involved in the regulation of some enzymes and metabolic pathways. Seed germination (with special reference to α-amylase regulation in cereal seeds, and dormancy break) and stem elongation are the two notable processes affected by GAs. Both have been studied with respect to changes in polyamine content and biosynthesis.

The specific activity of ODC in barley-seed extracts was found to increase with time of germination up to about 100 hr following imbibition, and GA_3 (as well as IAA) induced a fourfold increase in the activity.[28] Maximal induction of ODC activity was attained at 10^{-5} M GA_3, and higher concentrations brought ODC activity to control levels. Cadaverine also stimulated ODC activity of germinating barley seeds, whereas Put and Spd considerably inhibited ODC activity, indicating the induction of a protein inhibitor (antizyme) of ODC in plant tissues, similar to the situation in mammalian cells and in prokaryotes. Some interesting interactions of GA_3 and this antizyme were observed in the germinating seeds: addition of polyamines did not significantly affect ODC activity which was first induced by GA_3, but simultaneous addition of GA_3 and polyamines resulted in control levels. However, addition of GA_3 to seeds which were germinated for 50 or 98 hr in the presence of polyamines abolished the observed inhibition of ODC activity. Further studies of ODC activity in germinating barley seeds revealed the presence of a chromatin-bound ODC, the specific activity of which is 150 times higher than that of the cytosolic enzyme, and accounts for about 75% of total ODC activity at 120 hr of germination.[42] Both the cytosolic and the chromatin-bound enzymes were induced by GA_3 added at the start of germination, and further superinduced by addition of actinomycin D at 90 hr. Moreover, combined addition of GA_3 and actinomycin D facilitated the solubilization of the chromatin-bound ODC. Although these studies clearly indicate the effect of GAs on ODC activity, they were performed with fully germinated whole seeds and do not provide data on the interaction of polyamines with the direct effect of GAs on induction of α-amylase activity in the absence of cell division and growth. This aspect was investigated recently in GA_3-activated aleurone layers of barley seeds, where polyamine levels, ODC and ADC activities, and arginine and ornithine incorporation were studied.[43] Active polyamine metabolism, as revealed by increased polyamine levels and incorporation of precursors, occurs in the aleurone cells during seed germination. Addition of GA_3, however, did not affect polyamine levels in the aleurone during the 8- to 10-hr lag period of α-amylase induction by GA, nor did it affect the incorporation of arginine and ornithine into Put or the conversion of 1,4-^{14}C-Put to Spd or Spm. Likewise, treatment of aleurone layers with GA_3 for 2 hr resulted in no significant change in either the total or specific activities of ODC and ADC. In addition, this study showed that MGBG considerably inhibited both the GA_3-induced α-amylase activity and Spd content in the aleurone layers, and the inhibition of α-amylase by MGBG could be either completely or partially overcome by exogenous Spd. These results indicate that early action of GA on induction of α-amylase in barley seeds, in the absence of cell division and growth, is not on polyamine metabolism. The observed interaction with MGBG, as well as additional data,[44] suggests, however, that polyamines particularly Spd, may be involved in the regulation of α-amylase by GAs. In contrast to the lack of effect of DFMA and DFMO in the former study, other preliminary data seem to suggest that GA_3-induced α-amylase is inhibited by DFMA.[45] Some interactions of GA_3 and polyamines were also observed in other seeds: GA_3 (in addition to other hormones) reversed the inhibitory effect of KCl on ADC activity in cucumber cotyledons,[34] and ADC activity of rice embryos was considerably stimulated by GA_3.[29]

In addition to the studies of polyamine interactions with GAs in seeds, the effect of GAs on polyamine content and biosynthesis was also investigated in growing stems and apical buds. Growth of the terminal bud or internodes of pea seedlings responds to red light (phytochrome control) and GA in a different way, and this system was used for studying the interactions of GA with polyamines.[32,46-48] Spraying light-grown dwarf Progress pea seedlings with GA caused a marked increase in stem growth as well as a threefold increase in ADC activity in the fourth internode, whereas ODC activity was inhibited.[46] GA_3 treatment also resulted in a marked increase in the content per organ of Put and Spd (and less so of Spm) parallel to its effect on growth, showing a close correspondence between internode growth and polyamine content. Spraying ammonium (5-hydroxycaryacryl) trimethylchloride piperidine carboxylate (AMO1618), which reverses many of the GA effects, caused opposite effects to those of GA_3 with respect to growth, enzyme activity and polyamine content. In addition to GA_3 effects in light-grown dwarf peas, GA completely reversed the red light-induced growth inhibition and ADC activity in etiolated Alaska pea epicotyls, and had no effect on dark-grown plants. Another study[48] showed that the stimulation of ADC by GA_3 in light-grown peas appeared within 3 hr after its application, with two peaks of stimulation at about 9 hr and 27 hr after application (possibly prior to growth stimulation), suggesting a circadian rhythm and possibly implicating that the effect of GA_3 on ADC activity precedes its effect on growth. In an attempt to separate the light and hormone effect on ADC activity from those on growth, excised epicotyls of dark-grown Alaska pea seedlings were used.[47] Excised long epicotyls evinced growth promotion of buds after red-light irradiation, whereas excised short epicotyls did not. Red irradiation, however, induced an increase in ADC activity in buds of both groups, indicating that the light effect on ADC is probably independent from its effect on growth. In the same experimental system, GA_3 prevented promotion of both growth and ADC activity in long epicotyls by red light, as well as preventing the red light-induced increase in ADC activity in short epicotyls. Thus, the inhibitory effect of GA_3 on ADC activity in etiolated Alaska pea seedlings seem to be independent from its effect on growth. In contrast to former results with light-grown dwarf peas,[46] other data show that the promoting effect of GA_3 on polyamine accumulation is not always coupled with its effect on enzyme activity. Thus, spraying of 6-day-old mung bean seedlings with GA_3 resulted in a considerably increased concentrations of Put, Spd, and Spm in the subapical zone, and had no effect on ADC and ODC activity.[4] In older plants (10 and 12 days), however, GA_3 considerably inhibited ADC activity (and less so ODC activity) and slightly promoted polyamine accumulation. In a recent study on the effect of ethylene on growth and polyamine metabolism in deep-water rice,[49] (see details under Ethylene) it was found that GA_3 also enhanced ADC and SAM decarboxylase activity, and inhibited the decline in Put and Spd levels. A detailed study of pea lines of various internode phenotypes revealed yet another aspect of the interaction between polyamine and GA-induced growth.[50] The amounts of polyamines per organ in the different genetic lines, which differed in internode length and extractable GA content, correlated with the extent of internode elongation. By contrast, similar polyamine concentrations (per fresh weight) were often found in internode with extreme differences in final size. Thus, extremely short internodes of the ‘‘nana’’ line plants without GA exhibited equal or higher amine concentrations relative to other lines and GA-stimulated nana plants. The addition of GA_3 to nana plants increased the polyamine content but decreased polyamine concentration on a weight or length basis. High polyamine concentrations were usually associated with young internodes and decreased with internode expansion. DFMA and DFMO, either alone or in combination, partially inhibited polyamine accumulation and internode elongation of the tall pea line and GA-stimulated nana plants. These data indicate that polyamines do not mediate the entire action of GA in pea internode growth, which involves both cell division and cell elongation. However, the involvement of polyamine in cell division (in this study and others) suggests that polyamines may be needed for the full expression of GA-induced growth. The effect of GA_{20} (an endogenous GA in peas) on delaying senescence of the apical bud of G2 pea line has also been investigated recently.[51] Exogenous application of GA_{20} delayed senescence, enhanced bud size, and resulted in elevated Spd concentrations in buds, with no effect on Put and Spd levels. This may indicate that polyamines are required for bud growth, but are not directly involved in apical bud senescence in peas. Another study found that the GA effect on hypocotyls of lettuce seedlings may be partially mediated by polyamines, since applied Put, cadaverine, agmatine, arginine, and ornithine apparently enhanced GA-induced elongation.[52] A study of the effect of paclobutrazol, an inhibitor of GA biosynthesis, also suggests that a certain level of endogeneous GA may be required for maintaining polyamine concentration within plant tissues.[53] Paclobutrazol decreased endogenous Put and Spd concentration in apple seedling leaves, as well as leaf growth and water loss. GA_3 counteracted the inhibitory effect of paclobutrazol on polyamine content and leaf growth, which points to a correlation between leaf growth and polyamine levels. Water stress-induced accumulation of Put, and especially of Spd, were also prevented by paclobutrazol, which retarded the rise in ethylene production and two related metabolites. DFMA, but not DFMO, inhibited the rise of Put and Spd in water-stressed apple leaves (see more details under ‘‘Ethylene’’).

Although several detailed studies are available, the former data remains inconclusive and sometimes contradictory. The type of interaction between GAs and polyamines seems to be dependent on the investigated plant system, and is related to some very specific tissues and physiological processes. Thus, although polyamines seem to have a role in the phytochrome-controlled growth of peas and in the related GA effects, their causal relation to GA-induced activity of α-amylase is not clear. Likewise, the elucidation of polyamine function in GA-induced stem elongation still awaits further investigations.

D. Ethylene

The contrasting effects of polyamines and the plant hormone ethylene already attracted attention in 1981,[41] when it was hypothesized that the ''antisenescence'' effect of polyamines in wounded and detached plant organs is related to several possible mechanisms. The three suggested mechanisms included membrane stabilization, nucleic acid metabolism (especially inhibition of RNase activity), and inhibition of ethylene production, due to possible competition on SAM which is the precursur for both polyamines (Spd and Spm) and ethylene.[5,41] Since then, several studies have confirmed the suggested close interaction between polyamines and ethylene. Most of these studies deal with the effects of exogenously applied polyamines and related compounds on several aspects of ethylene production and biosynthesis, and only few examine how exogenous ethylene interferes with polyamine levels and biosynthesis.

The polyamines Put, cadaverine, Spd, and Spm considerably reduced ethylene production by senescing petals of *Tradescantia*, and inhibited the auxin-mediated ethylene production by etiolated soybean hypocotyls to less than 7% of the control.[31] This marked inhibition was attained at rather high polyamine concentrations (1 to 10 m*M*), although significant inhibition was observed at >0.1 m*M* Spd. In addition to their effect on the normal course of ethylene production, Spd and Spm also inhibited production of ethylene from the immediate ethylene precursor ACC, and reduced the endogenous content of this precursor. The authors regard this as an evidence that polyamines inhibit all steps of ethylene production. A marked inhibitory effect of Spd and Spm on ethylene production was also demonstrated in disks and protoplasts from apple fruits, and in Pinto bean and tobacco leaves.[30] These polyamines inhibited the normal course of ethylene production by apple fruit protoplast and tissue, as well as the conversion of ^{14}C-3,4 methionine to labeled ethylene in fruit tissue. In addition, the polyamines inhibited the auxin-induced ethylene production and the conversion of ACC to ethylene in leaves. The observed effects were attained at Put, Spd, and Spm concentrations of 1 to 10 m*M*, and Ca^{++} in the incubation medium reduced the inhibitory effect of the polyamines. Binding of polyamines to membranes, resulting in conformational changes which impair the functionality of the ethylene-synthesizing membraneous system, was suggested as the underlying mechanism. This assumption was tested, in part, in relation to the effect of polyamines on apple microsomal membrane microviscosity.[54] Spm and Ca^{++} both inhibited ethylene production in apple fruit disks, and lowered the temperature-induced rise in microsomal membrane microviscosity. The Spm effect on inhibition of ethylene production, however, differed from that of calcium: as the fruit ripened, inhibition by Spd decreased while that of Ca^{++} increased, and the inhibition by Ca^{++} was transitory while that of Spm persisted. In addition, the difference in the temperature-dependent inhibitory effect of Ca^{++} and Spm were probably related to membrane microviscosity: Spm, unlike Ca^{++}, stabilized membrane fluidity of apple fruit tissue above 4°C, thereby preventing the natural increase in microviscosity which normally accompanies tissue senescence with the rise in incubation temperature. These possible explanations of the nature of the interaction between polyamines and the ''senescence hormone'' ethylene, which are based on the assumption that polyamines interfere with membrane structure and functions, were further extended to leaf senescence. Spd and 1,3-diaminopropane, which retard cereal leaf senescence, inhibited the normal course of ethylene production in peeled oat leaves. Moreover both polyamines and ethylene biosynthesis from methionine was inhibited by inhibiting ACC synthase activity, and, more effectively, the conversion of ACC to ethylene.[55] In addition, 1,3-diaminopropane also decreased tissue levels of Put and, to a lesser degree, of Spd.[56] Ca^{++} competitively reduced the polyamine-mediated inhibition of ACC conversion to ethylene and chlorophyll loss with leaf senescence, suggesting attachment to common sites on membranes. Since ethylene did not reverse the effect of polyamines on chlorophyll loss, and as inhibition of ethylene biosynthesis by AVG did not prevent chlorophyll loss, the authors concluded that the inhibition of chlorophyl loss by polyamines in senescing leaves does not result from the inhibition of ethylene formation. Rather, polyamines probably attach to membranes, modify their structure and functions, and by so doing they independently retard ethylene biosynthesis and various senescence processes.

The suggested ''competition'' on SAM between polyamines and ethylene[5,41] is an alternative explanation for their interaction. In an experiment with aged peel disks of orange,[57] it was found that AVG, which inhibits ethylene biosynthesis at the ACC synthase site, stimulated incorporation of radioactivity from [3,4-^{14}C]-methionine into Spd. This may suggest that by inhibiting ethylene biosynthesis, the SAM pool became more available for polyamine biosynthesis. Exogenous Put and Spd, alone or in combination with AVG, also inhibited the conversion of methionine to ACC and stimulated incorporation of label from methionine into Spd, but this is probably related to a different mechanism. The suggested pivotal role of SAM pool in the control of the ethylene and polyamine pathways has been given additional support in a recent study with healthy and virus-infected Chinese cabbage protoplasts.[58] Inhibition of ACC synthesis from [3,4-^{14}C]-methionine by AVG in protoplasts, resulted in a twofold increase in both Spd and Spm. AVG permitted an increase in SAM, which is usually present in very low levels in the protoplasts, indicating that the accumulated SAM can, indeed, be utilized for polyamine biosynthesis. Inhibition of Spd synthesis in protoplasts by cyclohexylamine, CHA (a potent inhibitor of Spd synthase) permitted accumulation of small amounts of decarboxylated SAM, slightly inhibited the incorporation of radioactivity from methionine to ACC, and induced almost a twofold increase of incorporation into Spm. The interaction between polyamines and ethylene in water-stressed apple leaves was also studied in relation to paclobutrazol[53] (for further details see Section C). Although both polyamine accumulation and ethylene production were enhanced by water stress, they do not appear to compete with each other on SAM, which was maintained at a steady level, even when both were actively synthesized.

Inhibitors of polyamine and ethylene biosynthesis also proved useful for studying the role of polyamine metabolism in regulating ethylene production and senescence of cut carnation flowers.[59] Endogenous concentrations of Put (but not of Spd and Spm) increased considerably as flowers aged, parallel to a sharp rise in ethylene production. D-Arginine and DFMA greatly decreased Put levels of cut flowers, and these two inhibitors, and MGBG, promoted ethylene production together with the onset of senescence. Moreover, the ethylene biosynthesis inhibitor AOA caused a marked delay in the onset and progress of senescence, completely inhibited ethylene production, and induced a marked rise in Spm level. These results led the authors to propose that competition for SAM by the ethylene and polyamine pathways provides some degree of regulation during senescence of carnation flowers. The possible role of polyamines in senescence of the apical bud of G2 peas was also investigated recently.[51] In this study it was found that ACC (which increases ethylene formation) strongly decreased bud size, together with a steady decline in polyamine levels and a sharp drop in their concentration (together with apical senscence) at 10 m*M* ACC. On the other hand, AVG (which inhibits ACC synthesis) produced a marked increase in Put concentration in apical buds, but did not affect their growth. In contrast with the former interpretations of the interaction between polyamines and ethylene, and with previous evidence that polyamines may support nucleic acid and protein synthesis in senescing protoplasts,[60] data for apple fruit tissue may provide an additional interpretation.[61] Exogenous polyamines at concentrations of 0.1 to 10 m*M* markedly inhibited ethylene production, as well as uridine and leucine incorporation into macromolecules, which suggests that polyamines inhibit ethylene formation by inhibiting *de novo* protein synthesis (possibly ACC synthase).

The effect of ethylene treatments on changes in endogenous polyamines and their biosynthetic enzymes has been studied only recently.[62] Exposure of etiolated pea seedlings to physiological concentrations of ethylene greatly reduced the specific activity of ADC, provoking a fivefold increase in the Km of the enzyme and a tenfold reduction of its Vmax.

This ethylene-induced inhibition of ADC was completely reversible within 7 hr after transferral of seedlings to ethylene-free atmosphere. Moreover, treatments which reduced the endogenous level of tissue ethylene, or interfered with ethylene action, caused a gradual increase in the specific activity of ADC in the apical portion of pea seedlings. It is thus possible that both exogenous and endogenous ethylene affect polyamine levels by controlling ADC activity. In another study,[32] high concentrations of ethephon, an ethylene-releasing compound, inhibited ADC activity in excised terminal buds of etiolated peas following exposure to red light. All previous studies examined polyamine levels and metabolism in exposure to red light. All previous studies examined polyamine levels and metabolism in plant systems where ethylene inhibits growth or is associated with senescence. In a recent study with deepwater rice, where ethylene promotes cell division and growth in the internodes, it was found that treatment of stem sections with ethylene (and with GA_3) enhanced the activities of SAMDC and ADC and inhibited the decline in the levels of Put and Spd levels.[49] Submergence of rice plants, which leads to enhanced ethylene production, also brought about increased SAMDC and ADC activities (with no effect on ODC activity) and elevated levels of endogenous Put and Spd. The parallel increase in ethylene and polyamine biosynthesis in submerged rice plants may indicate that the two processes do not compete for a common substrate. The interrelationship of ethylene and the activity of ADC and ODC was also studied in citrus leaves in connection with ethylene-induced abscission. Ethylene (but not 2,4-D) caused a marked increase in the activity of ADC and ODC in the abscission zone, and it was suggested that the rise in ADC activity may have been caused by endogenous formation of new ethylene. These results indicated a connection between the increase in enzyme activity and ethylene treatment, but a significant relationship with the abscission process could not be established.[63]

Although the interaction between polyamines and ethylene has been given substantial attention, and seems to be the most studied plant hormone-polyamine system, the precise nature of this interaction has yet to be elucidated. Inasmuch as available biochemical data are concerned, competition between polyamine and ethylene biosynthetic pathways on SAM does not seem to play a pivotal role without confirmation that the SAM pool (which serves for many biochemical reactions) is indeed limiting. The role of the suggested alternative mechanism of ethylene-polyamine interaction, i.e., structural (and possibly functional) modification of nucleic acids and cell membranes, also must be elucidated. Thus, the term ''membrane stabilization'' is often used by the same investigators to imply both inhibition of membrane disruption and tissue senescence, as well as impairment of normal membrane functions.

E. Abscisic Acid

Of the major plant hormones, interaction of ABA with polyamines has been studied least. A recent investigation with imbibed barley seeds,[44] which have been previously studied in relation to GA-polyamine interaction (see also Section C) shows some effect of ABA on polyamine metabolism. Imbibition of embryonectomized half seeds of barley with ABA had no significant effect on the incorporation of label from L-[U-^{14}C]-arginine into free Put and only slightly inhibited incorporation into Spd. In the aleurone layer of seeds, however, ABA caused a small increase (less than 40%) in the content and incorporation of ^{14}C into the free form of Put. At the same time the ABA treatment resulted in 50 to 70% decrease in free Spd content and in ^{14}C incorporation into Spd. The author interprets this to suggest that ABA may directly or indirectly inhibit the conversion of Put to Spd, but not of Spd to Spm. This is however, in contrast with a former study,[43] in which ABA did not alter polyamine levels. MBGB has a marked and significant effect on polyamine metabolism in barley seeds when compared with the relatively small response to ABA.

While studying the interaction between cytokinins and polyamines in cucumber cotyledons[34] (see also Section B), it was noted that ABA inhibited cotyledon growth along with about 50% inhibition of ADC specific activity, an effect which was overcome by cytokinins. Similarly, a study with rice seeds revealed a decline in the total content of polyamines as well as a slight inhibition of ADC activity in ABA-treated seeds.[29,39]

IV. INDIRECT, PUTATIVE INTERACTIONS WITH PLANT HORMONES

The former data provided *direct* evidence for the interactions of plant hormones and polyamines, i.e., the effect of hormonal treatments on changes in endogenous polyamines, and (in a few cases) the effect of applied polyamines on changes in endogenous hormones. In addition to this direct experimental approach, changes in endogenous polyamines and effects of applied polyamines were also followed during developmental processes which are known to be normally regulated by plant hormones. This includes growth and morphogenesis of plant tissue and cell cultures, other growth and development processes, and effects of senescence and stress conditions. Thus, data from such experiments may provide circumstantial evidence for the interaction of plant hormones and polyamines. Selective examples of such interactions are presented in the following.

A. Tissue and Cell Cultures

Since growth and differentiation of plant tissue and cell cultures is dependent on appropriate relative concentrations of hormones in the medium (especially auxins and cytokinins), their interactions with polyamines are more meaningful than in other cases. Induction of embryogenesis in carrot cell suspension cultures, by transfer from auxin containing medium to an embryogenic hormone-free medium, was accompanied by a twofold increase in Put and Spm levels, whereas Spd levels were lower in embryogenic cells.[64] Incorporation of arginine into Put, as well as ADC activity, were also doubled in embryogenic cultures already 6 hr after transfer to hormone-free medium, suggesting that Put biosynthesis and polyamines may play a role in cellular differentiation during embryogenesis.[65] A requirement for polyamines and ADC activity during embryogenesis of carrot cultures was established by the use of inhibitors of ADC and of polyamine biosynthesis.[66,67] DFMA considerably inhibited embryo formation by inhibiting ADC activity, and Put and Spd concentrations in the treated cells were greatly reduced. Addition of Put, Spd, or Spm to the culture medium restored embryogenesis in the DFMA-treated cells. Spd synthesis inhibitors, MGBG and CHA, were also found to reduce growth and embryogenesis in carrot cultures concomitant with a reduction of Spd levels. In the same study, high concentrations of MGBG inhibited callus growth and, especially, shoot formation in cultured aspen hypocotyls. In a similar study, embryogenic cultures of carrot showed a temporal increase in Put, and especially in Spd and Spm levels, as compared with nonembryogenic cultues (where 2,4-D was not removed).[68] DFMA and CHA inhibited both the increase in Spd and Spm and embryogenesis, and the effect was reversible with Spd. Suppression of ADC and SAMDC activities of carrot cultures by auxin was also found, and this effect was possibly related to embryogenesis. The observation that a mutant cell line which failed to form embryos (but grew at the same rate as the wild type), also failed to show an increase in polyamines levels seems more convincing, thus indicating that the increase in polyamine content was associated with embryogenesis. The interaction of 2,4-D and polyamines in carrot embryogenesis was further pursued, and it was found that inhibition of Put synthesis via the ODC pathway by using DFMO induced somatic embryogenesis even in the presence of otherwise inhibitory concentrations of 2,4-D.[69] DFMA, on the other hand, inhibited growth and embryogenesis both in the presence and absence of 2,4-D, indicating that polyamines and ADC activity play a role in growth and differentiation of carrot cell cultures. Put and arginine in the culture medium of carrot cultures slightly affected embryogenesis, and it was claimed that Put inhibited the formation of globular embryos whereas arginine inhibited the subsequent development of the already formed globular embryos.[70]

Involvement of polyamines in differentiation of cultures other than carrot was also detected in a few cases. *Passiflora* leaf disks were cultured on media with modified hormone composition, which induced either callus, shoot, or root formation and a marked increase in Put levels was found to precede both callus proliferation and organogenesis.[71] Shoot and root formation in these cultures was also accompanied by a large increase of agmatine iminohydrolase activity, which participates in Put synthesis in the ADC pathway. Leaf explants of *Nicotiana tabacum*, which were cultured on shoot-forming medium (supplemented with cytokinins), accumulated one aliphatic amine (Put) and one aromatic amine (phenethylamine), but neither of these accumulated in the absence of cytokinins when roots were formed.[72] Other aspects of polyamines in cell and tissue cultures include the effect of added Put and changes in endogenous polyamines of *N. sylvestris* cell cultures,[73] changes in free and conjugated polyamine content of Paul's scarlet rose suspension cultures[74] and in tobacco cell and callus cultures,[75-77] selection of *N. tobacum* mutants which are temperature-sensitive or resistant to MGBG and evince altered polyamine biosynthesis and accumulation,[78,79] resistance to Put in a carrot callus line,[80] and DFMO-resistance in tobacco cell lines.[81]

In addition to their effect on growth and morphogenesis, polyamines are involved also in some aspects of protoplasts and cell senescence in culture. Rapid deterioration of *Avena* protoplasts was considerably retarded by polyamines, which stabilized protoplasts against lysis, inhibited the rise in ribonuclease activity, and supported protein and RNA synthesis.[60] Subsequent studies indicated that treatments of *Avena* protoplasts with polyamines promotes DNA synthesis and the inception of mitotic activity, although normal callus growth has not been achieved.[82] Likewise, addition of ornithine and Put to the culture medium of *Alnus* protoplasts stimulated cell division and colony formation, and subsequent active callus growth.[83] In addition to their effects on protoplasts, diamines and polyamines in the culture medium were found to inhibit senescence (i.e., increase viability) of nonphotosynthetic Paul's scarlet rose cell suspensions.[84]

The above-mentioned data provide strong evidence that polyamine content and biosynthesis play a role in the control of tissue culture growth and morphogenesis in a few selected cases, and especially with carrot embryogenesis. As cell and tissue cultures are dependent on the presence of plant hormones in the medium, polyamines and plant hormones probably interact in some manner but, at this stage, the evidence is only circumstantial.

B. Other Growth Effects

The involvement of polyamines in the various aspects of plant growth and development is reviewed in detail in another chapter of this book. Of the many developmental processes, the following seem to be normally and directly controlled by hormones and are thus relevant to our discussion: seed germination and seedling growth, root and shoot growth, fruit set, and development. Indeed, major changes in polyamine content and biosynthesis were observed in these cases. Selective examples include seed germination and seedling growth of bean and pea,[85,86] *Lathyrus*,[87] corn,[88] and rice,[89] and actively growing stems and roots of bean, tomato, potato, and corn.[90-93] The participation of polyamines in fruit set and development seems of special interest in view of its dependence on pollination, the most important single stimulus (which can be replaced by auxin treatment in many cases). This has been studied in detail in relation to tomato fruit set and development,[94,95] tobacco ovary maturation,[96] apple fruit set,[97] and development and ripening of avocado and citrus fruits.[98,99] It was found recently that lateral root differentiation and development in corn, a typical hormone-regulated process, is dependent on polyamine content and biosynthesis, and ODC activity was localized in actively dividing meristematic cells which give rise to lateral root.[100] These, and a few other examples, support the hypothesis that plant hormones and polyamines interact, in an as yet unknown manner, in the control of several growth processes in plants.

C. Senescence and Stress

A detailed review of polyamine involvement in senescence and stress is presented in another chapter of this book. Most types of stress responses (e.g., osmotic, acid, salinity, wounding) have little to do with hormonal regulation (but see Section III.C). Leaf and flower senescence, however, is contradistinctive in that it is associated with hormone action, with special reference to ethylene and cytokinins. Thus, several parameters of leaf senescence, such as chlorophyll breakdown, protease activity, and structural changes in chloroplasts, can be delayed by cytokinins and promoted by ethylene. The effects and changes in polyamines during leaf senescence, indeed seem well established, and are supported by several publications, commencing with the finding that arginine, ornithine, and lysine retard oat leaf senescence,[101] and that polyamines are able to stabilize and delay the senescence of oat leaf protoplasts.[60] The effect of polyamine treatments on delaying senescence of excised leaves has now been demonstrated in a wide range of plant species. The senescence-linked events which are retarded by polyamines include chlorophyll breakdown, protease and RNase activities, and accumulation of α-amino nitrogen and stabilization of thylakoid membrane functions.[102-108] In several cases, it was found that polyamine content and activity of ADC and ODC decrease progressively in excised as well as in intact senescing leaves.[5,103] It should be mentioned, however, that in some cases exogenous polyamines did not retard certain parameters of leaf senescence.[109] Kinetin and GA_3 were also found to accelerate Put loss in senescing pea leaves while retarding the loss of Spd and Spm.[109]

Unfortunately, direct interaction of polyamines and hormones in delaying leaf senescence was not investigated (except for limited studies concerning ethylene), and causal connection between the two was not established.

V. COMPARATIVE FUNCTIONAL CHARACTERISTICS OF PLANT HORMONES AND POLYAMINES

A short comparison of plant hormones and animal hormones will be presented first, followed by a discussion of several characteristics of plant hormones and polyamines, which may be relevant to their functions.

A. Plant vs. Animal Hormones

Plant hormones differ from animal hormones in relation to both functions and chemical structure:

1. Plant hormones are low molecular weight organic compounds (molecular weights, in ascending order): ethylene — 28, IAA — 175, benzylaminopurine — 225, ABA — 264, and GA_3 — 347. Although the molecular weights of a few animal hormones may be close to those of plant hormones, they are structurally more complex (i.e., steroids or peptides).
2. In contrast to animal hormones, plant hormone production is not confined to specialized glands and cells, and they are formed by almost all living cells and tissues. In some cases, however, high production rate of plant hormones can be correlated with actively dividing, meristematic tissues. Directional transport of plant hormones (otherwise referred to as "polar transport") is, thus, not a prerequisite for plant hormone functionality under all conditions — although long-distance transport of plant hormones does in fact occur.
3. The functions of a given plant hormone are not limited to a single specific effect or mechanism as is the case with most animal hormones. Rather, plant hormones control a broader spectrum of processes, and the varying effects of each of the plant hormones can be expressed differently in diverse plants or even in different tissues and organs

within the same plant. Moreover, specific physiological or developmental effects can be exerted by two or three plant hormones.

These differences between animal and plant hormones should be kept in mind when evaluating the following questions: Do polyamines act as plant growth regulators? Do polyamines function as second messengers of plant hormones? This aspect will be discussed in the following.

B. Endogenous Concentrations and Exogenous Treatments

Endogenous concentrations of free polyamines in plant tissues are higher than those of most hormones. The content of plant hormones is usually less than 1 μg/g fresh weight of tissue, except in specific cases (e.g., ethylene in ripening climacteric fruits and ABA in water-stressed leaves) where higher levels can be detected. Following are the prevalent ranges of polyamine levels (microgram per gram fresh weight): Put: 1 to 800, Spd: 1 to 250, and Spm: 0.5 to 150. This wide range represents different plant species, various types of tissues and organs, and different growth conditions and developmental stages. The ratio between Put, Spd, and Spm contents is also subject to modifications by these factors. It is thus obvious that in many cases tissue polyamine content is higher than that of plant hormones by at least one order of magnitude, and in some cases even by two to three orders of magnitude. When compared with the normal content of other cations in plant tissues (e.g., Ca^{++}, K^{+}, Mg^{++}), polyamines usually occupy an intermediate position between these cations and plant hormones (Ca^{++} and K^{+} are usually present at 1000 to 6000 μg, and Mg^{++} at 400 to 2000 μg/g fresh weight). This consideration may be of some relevance to the regulatory functions of polyamines.

Most of the effects of exogenously applied polyamines are attained usually at a concentration range of 10^{-3} to 10^{-5} *M* (sometimes even at 10^{-2} *M*) with many effects at 10^{-4} *M*. Plant hormones typically exert their effect when applied at lower concentrations, usually of 10^{-5} to 10^{-7} *M*. Tissue cultures and in vitro reactions are sometimes affected at lower concentrations of polyamines and plant growth regulators, and bioassays for plant hormones are normally sensitive to even lower concentrations of the hormones. The fact that higher concentrations of polyamines than those of plant hormones are usually required, may be attributed to the following factors: difficulties in penetration and/or transport to the site of action, rapid metabolism, lack of tissue sensitivity (i.e., physiological response is not dependent on polyamines), or that the endogenous polyamine concentrations are sufficient.

C. Uptake and Translocation

As mentioned previously, cell-to-cell and long-distance transport of plant hormones from zones of higher production rate (e.g., meristems) can be regarded as a regular feature of plant hormones, providing the basis for their role in whole plant development. The question of polyamine transport in plant tissues is, therefore, relevant to their hypothesized functions in growth regulation.

This issue was first studied in etiolated pea seedlings.[110] Injection of ^{14}C-labeled Put, Spd, arginine, and lysine to cotyledons resulted in only very little transport of Put and Spd to shoots and roots during 4 hr. Transport of lysine and arginine away from the cotyledons was, however, more significant, and a large fraction was recovered as cadaverine and Put. On the basis of these results, the authors concluded that the evidence for polyamine transport is inconclusive, and polyamines, therefore, cannot be regarded as hormones.[111] Recent studies, however, show that polyamines can be taken up by plant tissues, and are translocated over a considerable distance.[112] Put, Spd, and Spm uptake in excised petals of *Saintpaulia ionantha* occurred against a concentration gradient, or followed a concentration gradient, at low and high external concentrations of polyamines, respectively. In addition, the rate of uptake was constant for a few hours, pH dependent, modified by inorganic cations, and dependent to some extent upon membrane potential.[112,113] Furthermore, flowers and young leaves of apple trees absorbed exogenous Put and it was demonstrated that apple fruitlets and young leaves can synthesize polyamines and translocate Put via the peduncle.[114] Former experiments on the effect of salt stress on polyamines in mung bean plants suggested that high concentrations of NaCl induced Put biosynthesis in roots, which was then translocated and accumulated in the shoot.[115] This is supported by recent data showing uptake of radioactivity-labeled Put and Spd from intact roots and hypocotyl cut surfaces, and their subsequent translocation along the stem to leaves.[116] This translocation was concentration dependent and affected by environmental conditions which interfere with transpiration. Furthermore, Put and Spd were detected in considerable amounts in both xylem and phloem exudates of several herbaceous (mung bean, sunflower) and woody (citrus, grape) plant species, their concentration in the exudates being dependent on the physiological condition of plants.[117]

Thus, the uptake, intertissue, and interorgan translocation of polyamines in several plant species may further support their suggested regulatory function in plant growth and development.

D. Molecular Aspects

When discussing functional characteristics of plant hormones and polyamines, and their possible interactions, several molecular aspects seem especially important, and are mentioned briefly in the following.

Proteins and nucleic acids — In several cases, hormone applications result in increased protein and nucleic-acid synthesis in plant tissues, but often it is the result of increased growth and/or cell-division processes rather than their direct cause. Recent studies with a few selected systems show, however, that plant hormones exert their effect at the transcriptional and translational level. In few cases the appropriate, hormone-responsive, cDNA probes were prepared. The function of the binding of polyamines to proteins and nucleic acids in plants are even less understood. The few available studies show that polyamines can inhibit RNase and DNase activity (usually in senescing or damaged tissues,[5,102-104]) and indirectly support increased nucleic acid and protein synthesis,[60,82,118] and promote, to some extent, enzyme activity.[119,120] Work in progress is aimed at cloning the genes for plant ADC and ODC.[121] We have found also that polyamines inhibit the activity of several restriction endonucleases, when assayed in vitro with total chloroplast DNA and two cloned chloroplast genes.[122]

Binding and receptors — In contrast to animal hormones, data of specific binding of plant hormones to protein receptors are scarce and inconclusive. The situation for auxins, cytokinins, and ethylene may be somewhat clearer than for the other two hormones, but the role of the putative receptors remains to be elucidated. Polyamines bind electrostatically to nucleic acids of plants, but binding does not seem specific. In vivo binding of polyamines to nucleic acids was studied in some detail in the case of DNA and several RNA species of *H. tuberosus*, spinach, and pea.[19,123] As yet, no direct evidence of polyamine binding to plant membranes is available, although this is suggested, indirectly, by recent in vitro studies with bean leaf membranes, albeit with very high polyamine concentrations.[124]

Mutants and cell lines — Mutants deficient in hormone activity are quite rare in plants, with the exception of mutants which lack several key steps in ABA and GA biosynthesis. Unfortunately, true plant mutants for polyamine metabolism are as yet unavailable, with the possible exception of tobacco (as discussed separately in another chapter of this book). A few cell lines, which may be eventually useful for elucidating the functions of plant polyamines include resistance of sugar cane and soybean cells to canavanine,[125,126] wild-type and *p*-fluorophenylalanine-resistant tobacco cell lines which are altered in polyamine level and metabolism,[127,128] and a carrot callus line resistant to high Put concentrations.[80] In a recent study, we have isolated tobacco cell lines which are resistant to high concentrations of DFMO (and, possibly, DFMA) and are altered in polyamine content and metabolism.[81]

Metabolic diversion into alternative pathways — Put can accumulate in tissues in considerable amounts and be diverted into pathways other than Spd and Spm biosynthesis, notably to biosynthesis of cinnamoyl-putrescines,[75,128] plant alkaloids, and especially nicotine. This aspect was studied in several cases, mainly with tobacco plants, calli, and cell cultures.[77,129] Polyamines and polyamine precursors can thus be shunted into alternative metabolic pathways, resulting in the possible masking of interactions between given growth responses and polyamines. This is in contrast with plant hormones whose endogenous concentrations are very small, and are therefore not recycled in significant amounts into other metabolites.

VI. CONCLUDING REMARKS: A BIOREGULATORY ROLE FOR POLYAMINES?

Recent investigations confirm that treatment with plant hormones results in significant changes in polyamine content and biosynthesis, in many plant species and with respect to many developmental processes. Changes in hormone levels and synthesis in response to polyamine treatments have been studied only occasionally, and in relation to ethylene. The interaction of polyamines with auxins, GAs, and ethylene have received more attention than with cytokinins and ABA, and hence more experimental data are available for the former. The close interaction of auxins and polyamines has been demonstrated satisfactorily with respect to activation and growth of *Helianthus* tissue in vitro, root formation in mung bean cuttings, and postpollination events leading to fruit set in tomato and possibly apple. The type of interaction between GA and polyamines depends on the investigated plant system, e.g., a more direct one in the case of phytochrome-controlled growth of peas, and a less defined interaction with α-amylase activity in barley aleurone and with stem elongation. Polyamine treatment of senescing plant tissues inhibits, in many cases, the normal course of ethylene production and biosynthesis. Ethylene treatment was also found, at least in one case, to modulate certain aspects of polyamine synthesis. Polyamines and ethylene may interact metabolically (at the SAM level) or structurally (in relation to membrane integrity), but conclusive evidence to either possibilities is not available as yet.

In addition to direct evidence for polyamine changes in response to hormone application, and vice versa, major fluctuations in polyamines also occur during developmental processes which are normally regulated by plant hormones. Notable, among these, are growth and morphogenesis of tissue cultures, seed germination and growth of some organs, and certain aspects of senescence. The data of these investigations do not provide, however, sufficient direct evidence to a causal interaction between polyamine metabolism and plant hormones. In fact, it can be argued that polyamines and plant hormones change simultaneously, but are not causally related to each other. In some processes, (e.g., fruit ripening) hormone levels may fluctuate dramatically with little change in polyamines, in others (e.g., acid or salinity stress, and mineral deficiency) polyamines levels, but not hormone levels, are increased considerably, yet in others (e.g., seed germination) large fluctuations in the levels of both hormones and polyamines can be detected.

The wealth of recent experimental data on plant polyamines suggests that they have a bioregulatory role in plant development and response to stress, similar to that of recognized plant hormones. This assumption is based mainly on findings that polyamines may stimulate or modulate several developmental processes in the absence of hormone application, that hormone application results, in many cases, in significant changes in polyamine levels, and that polyamine biosynthesis may often increase prior to any observed change in growth or differentiation. Whether polyamines merely reflect changes in plant metabolism and hormone levels, which accompany growth and development, or are involved in the regulation of these changes, is a crucial question. A comparison of the different functional characteristics of plant hormones and polyamines indicates that, at present, it is difficult to ascribe to plant hormones any more regulatory functions than to polyamines. This applies to the lack of sufficient convincing data, with regard to plant hormones and polyamines alike, as to specific binding to receptors and regulation of gene expression, and as to the availability of useful plant mutants and cell lines. On the other hand, plant hormones and polyamines are both produced and metabolized by all cells, especially ''activated'' cells (i.e., actively dividing or differentiating cells, and stress-activated tissues), and are both translocated between tissues and organs. It is, thus, premature to regard polyamines as second messengers of plant hormones, until more specific molecular information is available. The different concentration range of exogenously applied polyamines and plant hormones, and of their endogenous content, may be the only major difference between the two groups of substances. This may be related to the most obvious specific characteristic of polyamines, but not of plant hormones, i.e., their cationic versatility resulting in a high affinity for acidic constituents, such as charged membranes and nucleic acids. It is thus possible that polyamines are able to modulate plant hormone action through a fundamental mechanism common to all living organisms. The considerations mentioned above, together with the fact that plant hormones differ from animal hormones in several major characteristics, suggest that polyamines can be classified as plant growth regulators.

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Chapter 10

POLYAMINES IN PLANT MUTANTS

Russell L. Malmberg and Andrew C. Hiatt

TABLE OF CONTENTS

I. Introduction 148

II. Mutants Directly Selected for Polyamine Alterations 149
  A. Selection Strategies 149
  B. Analysis of Specific Selected Lines 149

III. Analysis of Mutants Selected for the Other Phenotypes 152
  A. General Approach 152
  B. Analysis of Specific Mutants 152

IV. Regulation of Polyamine Biosynthesis Deduced from Variants 154

V. Conclusions 158

Acknowledgments 158

References 158

I. INTRODUCTION

Provocative hypotheses have been made as to the roles of polyamines and polyamine conjugates as plant growth regulators, based on correlations observed with such phenomena as the cell cycle, stress response, hormone response, and development (see Chapters 7 to 9 in this volume). The emergence and refulgence of plant polyamine metabolism as a recent field of study has the consequence that much is still unknown about the regulation of the pathway and its relative importance in the phenomena just mentioned. On the basis of purely physiological or biochemical data, it can be difficult to separate correlations from causation. If a given treatment of a plant causes a change in polyamine or polyamine-conjugate titer, how is one to deduce if this correlation has a profound physiological meaning, or if it is a less important secondary effect of whatever is really important and primary? Doubtless there are many approaches. In this chapter we review (and advocate) the examination of genetic mutations that alter polyamine metabolism.

The philosophy of a mutational analysis is that if a genetic lesion is identified, then the changes in the phenotype of the organism, how it differs from wild type, should be attributable to the genetic alteration. Thus, by comparing a mutant individual with wild type, it should be possible to deduce the normal functions of the gene that is altered in the mutant. In theory then, one should identify the genes that encode the proteins for the polyamine enzymes, find mutations that knock out or alter the nature of the enzyme, examine the biochemical consequences of the lesion on the polyamines themselves, and then conclude that any physiological or developmental changes in the mutant individual are a consequence of the original mutation. In practice, one usually begins with a mutant individual, and then attempts to deduce the structural gene alteration. If the mutant individual can be clearly shown to have a single genetic lesion, and if the lesion can be unequivocally demonstrated to alter a specific gene, then the deductions as to the function of the gene product are valid. One difficulty is that many genetic experiments begin with mutagenesis or with the crossing of different strains, so that great care is needed to demonstrate that the line being examined has only a single mutation, only a single genetic difference with wild type. A second difficulty is that biochemical characterization of a mutant will not always identify the structural gene that is altered by the mutation; thus the primary genetic lesion may be hard to identify. In practice, then, one must always ask how nearly these criteria have been approached, before making conclusions.

In this review, we will discuss two types of approaches to analysis of plant polyamine mutants. In the first approach (Section II) we review work in which an attempt is deliberately made to select mutations that alter polyamine metabolism, to directly obtain lines with an altered polyamine metabolism. The biochemical phenotype of these mutants is then studied, to determine the precise gene or DNA sequence that is altered, and subsequently the organismal phenotype, e.g., the development or stress response of the plant is examined. In the second approach (Section III), we review work in which initially a plant with an interesting organismal phenotype is identified, e.g., lack of hormone response, and then the biochemical phenotype is worked out, and the genetic lesion is hopefully identified. The subsequent deductions for both approaches are that the primary genetic event results in the biochemical level alterations in polyamine synthesis, and this must then be translated into the altered organismal phenotype, assuming that the mutant under analysis really does involve a primary defect in polyamine metabolism. In Section IV we attempt to integrate what has been learned about the regulation of polyamine metabolism by studies on plant cell cultures. From this kind of data one should be able to study both the genetic and biochemical regulation of polyamine metabolism, and also to rigorously test the involvement of the polyamines in plant growth regulatory phenomena.

II. MUTANTS DIRECTLY SELECTED FOR POLYAMINE ALTERATIONS

A. Selection Strategies

Many of the studies we describe here were performed on plant cell cultures. The lure and romance of these systems is that the plant cells can be grown on minimal defined media, that large numbers can be plated out for mutant selections, that cells can be transformed with various DNA vectors, and that whole plants can sometimes be regenerated from cultures (by appropriate hormonal manipulations) for organismal, developmental, and genetic analysis. One disadvantage is that all of these steps can as yet only be performed on a few plant species, such as tobacco, petunia, and carrot. Tobacco and petunia regenerate from cell culture directly into shoots with leaves, and then are rooted on a separate media; this method is known as organogenic regeneration. Carrot cell cultures are models of embryogenic regeneration, in which the cells in culture are induced to regenerate via sequence of events that mimics normal zyogtic embryogenesis.

Because plant cell cultures can be grown readily on minimal media, it is straightforward to test the effects of the polyamine synthesis inhibitors on the growth of the cells. If the inhibitor kills the cells, then a strategy can be devised to obtain mutant lines that are resistant to the inhibitor. Biochemical screening of the appropriate enzymes and polyamine levels in the resistant cell lines can then help to determine the mechanism of resistance, and hence allow one to make judgments as the nature of the mutation. Resistance to polyamine analogues and inhibitors has thus been one method of direct selection for lesion in the pathway.[1,2] A similar type of genetic strategy can be applied to isolate polyamine-resistant[3] or polyamine-utilizing cell lines.[4] The distinction between these is that the polyamine-resistant lines will survive a normally toxic dose of an exogenously applied polyamines while a polyamine-utilizing line will substitute the supplied polyamine in place of its normal nitrogen or carbon source. These types of mutants, resistances, and utilizations are straightforward to isolate, by positive selection, strategies because they are likely to be the result of dominant mutations, and hence can be isolated readily from cell lines that contain more than one copy of each gene.

A variety of negative selection strategies are also available for plant cell cultures in order to isolate lines of that type that cannot grow where wild-type cultures will grow, e.g., auxotrophs. These procedures have not generally been applied to the problem of obtaining auxotrophs or temperature-sensitive lines deficient in polyamine synthesis. One example exists[5] where a temperature-sensitive line was initially isolated for lack of growth, and then subsequently determined to have altered polyamine synthesis (see the discussion of *ts4* below).

B. Analysis of Specific Selected Lines

Selection for methylglyoxal-bis(guanylhydrazone) (MGBG) resistance[1,2] resulted in 31 *Nicotiana tabacum* (tobacco) cell lines that showed resistance to 10 m*M* MGBG. All of these lines were isolated from UV light-mutagenized cells, which were subsequently plated out onto medium containing the MGBG. No resistant lines were obtained from the non-mutagenized control. The estimated mutation rate in the mutagenized cultures was extremely low, approximately 8×10^{-8}. This suggests that MGBG resistance is biochemically, not an easy trait to obtain. It also means that special caution is needed in interpreting data from these lines, in that the possibility exists of a double mutation. Resistance to difluoromethylornithine (DFMO) has also been reported[6] in a similarly mutagenized tobacco culture. Recently, Bagni and Mengoli[3] have isolated a putrescine-resistant carrot cell line by serial passage of carrot cells on 1 m*M* putrescine Flores and Filner[4] have isolated a tobacco cell lines able to grow on putrescine as the sole nitrogen source. Finally, as mentioned previously, Malmberg[5] isolated a tobacco line temperature sensitive for growth, and subsequently its polyamine metabolism was examined; a revertant able to grow at high temperature was subsequently obtained[7] and its polyamine metabolism was also checked. In the paragraphs that follow, we examine what has been reported individually about each of these lines selected for biochemical alterations of polyamine metabolism.

ts4 — This line was isolated from tobacco by a bromodeoxyuridine negative selection scheme,[5] is pleiotropic in its biochemical effects, and has 4% of the wild-type levels of ornithine decarboxylase (OrnDc) and *S*-adenosylmethionine decarboxylase (SamDC). On SDS polyacrylamide gels, an extra band of 35,000 daltons is seen; wild-type cell cultures grown on sublethal levels of MGBG also overproduce this 35,000-dalton polypeptide.[1] We subsequently demonstrated that this extra polypeptide is SamDC,[8] so that *ts4* produces large amounts of an inactive SamDC. Recent preliminary results suggest that the *ts4* line also has an altered rate of turnover of SamDC lending credence to the idea that the primary genetic lesion might be in the SamDC gene or in a gene that directly modifies SamDC (Malmberg and Rose, unpublished results). This cell line regenerates into a weak, light green plant that is dwarf and that never flowers.[7]

Rt1 — *Rt1* is a revertant of *ts4*,[7] that is partially blocked in polyamine synthesis, having near wild-type levels of OrnDC activity, but reduced levels of SamDC activity. It does not overproduce the 35,000-dalton polypeptide. Plants regenerated from *Rt1* are nearly normal in color and size. The flowers are both male and female sterile, but with the abnormality that the anthers are partially turned into petals (petaloid anthers).

Dfr1 — Dfr1 is resistant to DFMO,[6] Enzyme assays of this line indicate that it has low levels of OrnDC, and the residual activity that it does have is partially resistant to DFMO in the enzyme assay cocktail. Previously, on the basis of preliminary data we had believed that the *Dfr1* OrnDC was sensitive to DFMO; however, more careful and thorough experiments have revealed that the *Dfr1* enzyme is in fact partially DFMO resistant. *Dfr1* regenerates into an extremely weak dwarf plant that never flowers. Physically it resembles the *ts4* mutant just described. Both *ts4* and *Dfr1* took an unusually long time to regenerate into plantlets, over a year for each, whereas wild type normally regenerates in 2 months.

Mgr3 — Mgr3 is resistant to MGBG,[1] and overproduces spermidine and spermine relative to wild type. Plants regenerated from *Mgr3* have the abnormality that their ovules are switched into stamen (stamenoid ovules), a female-to-male switch. Other MGBG-resistant lines that display the stamenoid ovule phenotype are *Mgr1*, *Mgr4*, *Mgr9*, and *Mgr31*.[6] The *Mgr3* mutant is male sterile with defective anthers as well. In one case we were able to obtain nine seeds from a cross of wild type to *Mgr3*. The drug resistance and floral phenotype cosegregated as a nuclear dominant trait within this small F1 generation. Two different assays for resistance to MGBG have been used in scoring the phenotype of F1 and advanced backcross generations. First, pieces of leaves have been placed back in culture, and then resistance is scored by growth of the cultured cells in the presence of MGBG; second, resistance can sometimes be tested by growing seedlings in the presence of MGBG; and then scoring for growth and survival. In one backcross generation, the *Mgr3* mutation behaved as if it were a maternal effect mutation for seedling resistance to MGBG.[35]

Mgr9 — *Mgr9* is resistant to MGBG.[2] In addition to stamenoid ovules, this line has regenerated into plants with stigmoid anthers, a male-to-female switch, with simple defective anthers. Other lines that display the stigmoid another phenotype are *Mgr1*, *Mgr12*, and *Mgr27*.[6]

Mgr12 — *Mgr12* resistant to MGBG.[2] In regenerated plants *Mgr12* is male sterile, and is a nuclear dominant mutation by meiotic genetic analysis.[2] Biochemical analysis of crude extracts of cultures established from heterozygous F1 plants showed that approximately 50% of the SamDC activity was resistant to the presence of MGBG in the assay cocktail. This suggests that the mutation may be in the structural gene for SamDC or in a gene whose product modifies SamDC. In preliminary experiments, approximately one fifth of the MGBG-resistant mutants show similar kinetic evidence of being a structural gene mutation.

Mgr21 — *Mgr21* resistant to MGBG.[2] Preliminary biochemical analysis indicates that this line has elevated spermidine levels and elevated SamDC activity levels, although the kinetics of the SamDC itself do not seem to be altered.[34] This plant differs from the bulk of the MGBG-resistant plants in that it is extremely dwarf. It flowers with a ''puzzle box'' or ''nested doll'' phenotype. When one dissects a normal tobacco flower, the sequence of structures is sepal, petal, stamen, and carpel. In *Mgr21* the carpel is a hollow cylinder with the stigma surface having a hole down the middle. Inside this hole, a partial floral sequence is repeated, including stamen and carpel. The mutant seems to partially alter the determinate character of the normal tobacco flower; the terminal meristem is no longer committed to developing into part of the carpel, but rather dedifferentiates into an earlier phase of floral development. It stutters. These flowers also show a partial conversion of sepals into leaves. *Mgr15* is similar to *Mgr21*.[6]

Putrescine resistance[3] — This carrot cell line was isolated by serial passage on medium containing 1 m*M* putrescine. This line has 13-fold higher than normal levels of putrescine, and shows a reduced putrescine uptake at low concentrations of putrescine in the media. The enzyme activities of OrnDC, SamDC, and ArgDC were all similar to the control cultures. Regeneration of this line and genetic analysis has not yet been reported.

Putrescine utilization — A tobacco cell line was selected by serial culture to grow on medium containing putrescine as the sole nitrogen source. Biochemical analysis of the line indicated that it efficiently converted putrescine to α-aminobutyric-acid (GABA), whereas wild type does not. This transition may be mediated by a cinnamoyl derivative, which would imply that the altered cell line has converted a normally secondary metabolite to a primary one.

It should be clear after consideration of these results that the direct mutational analysis of polyamine metabolism is in its infancy. A first question that can be asked is if the structural genes for any of the enzymes have been identified as altered in the mutant lines. There is some evidence that *Dfr1* has an altered OrnDC enzyme, in that its activity is partially resistant to DFMO; however, given the likely existence of posttranslational modifications and control mechanisms, there is certainly a good chance that the mutation might fall into a gene that encodes an OrnDC modifying enzyme. Similar evidence exists that several of the *Mgr* lines (approximately one third of them) might have altered SamDC on the basis that the enzyme is partially resistant to MGBG in the assay cocktail, and the same argument holds that the mutational event could be in the gene for a modifying protein as well as it could be in SamDC structural gene itself. Recent unpublished data on the *ts4* line also suggests it might be altered in either the structural gene for SamDC or in a gene that sets turnover rates, since the *ts4* line may have a reduced rate of turnover of SamDC. The putresine-resistant carrot line has been demonstrated to have altered putrescine uptake at low levels of exogenous putrescine, so it is reasonable to hypothesize that there is an alteration in a membrane protein that functions in putrescine transport.

Do these variant lines tell us anything new about the regulation of the polyamine pathways in plants? The existence of cell lines that change polyamine concentrations, such as *Mgr3* without detectable alterations in the main synthetic enzymes, suggests that there are genes whose functions are to regulate the pathway. However, until further biochemical and genetic work clearly defines the precise nature of the lesion in these lines, it is difficult to make a statement about whether or not a mutant defines a regulatory gene. Obviously one cannot define the nature of a mutation by merely demonstrating a lack of changes in the proteins tested.

Because of the many alterations of the floral morphology that have occurred, many of the polyamine mutants are sterile, particularly the *Mgr* series. However, a preliminary genetic description of *Mgr3* and *Mgr12* is available as nuclear dominant mutations, in which only a single locus seems to be segregating for resistance. There is no substitute for a genetic analysis to sort out whether or not a given phenotype may have been caused by a double mutation instead of a single event. It will also be extremely important to try to construct double mutant lines in order to perform complementation analyses and to study the interactions among the mutant phenotypes.

III. ANALYSIS OF MUTANTS SELECTED FOR THE OTHER PHENOTYPES

A. General Approach

Because of the many interesting phenomena in which polyamines have been implicated, an effort has been made in a number of laboratories to examine polyamine metabolism in plant mutants which were initially selected for having an interesting developmental or physiological phenotype. For example, since a role in floral development has been postulated for polyamines and polyamine conjugates, Martin-Tanguy et al.[9] and Dumas et al.[10] have studied floral mutants of maize and tobacco. Similarly, since polyamines have been implicated in stress response, Shevyakova et al.[11] have studied polyamine metabolism in NaC1-adapted cell lines of *Nicotiana sylvestris* (a wild relative of cultivated tobacco). In this section we will review these papers and others where the approach has been to begin with a preexisting, interesting, plant mutant, and then analyze it biochemically.

The logic clearly is that if polyamines are to be implicated as a growth regulator in a given physiological process, then a whole plant mutant that is deficient in that process should show some alterations in polyamine metabolism. This is not a strict requirement in the sense that a given developmental or stress signal may initiate a chain of events, one link of which is the alteration in polyamine metabolism. Thus, if the mutation is in fact further down the chain of events than the polyamine step, then there may be no alterations seen, and the lack of such alterations would not disprove the possible role of polyamines in that process. By this logic, however, some of the mutants for a given phenotype must include alterations in polyamine metabolism. Conversely, the finding that there is a change in the pathway in a given mutant plant does not constitute proof that the alteration in polyamines is necessarily important to the physiology and whole plant phenotype of that mutant. What is needed is a clear definition, in biochemical terms, of the actual primary genetic lesion of these mutants. After the discovery of the primary biochemical defect, then the proper role of the polyamines in the whole-plant phenotype can be property assessed.

B. Analysis of Specific Mutants

Several laboratories have analyzed gibberellic acid (GA) dwarf mutants of *Pisum sativum* (peas) in order to examine the possible role of polyamines in plant hormone response. Dai et al.[12] have examined a dwarf pea variety, after treatment with GA to cause internode elongation. They found that the activity of ArgDC, and the levels of putrescine and spermidine, all rose in parallel with the promotion of internode elongation of GA. Further, the use of a partial GA antagonist prevented internode elongation and also prevented the rise in ArgDC and polyamine levels. The content of polyamines per gram of material remained constant, but the content per stem section rose after internode elongation. Smith et al.[13] have carried this type of analysis further, by extending the measurements of polyamine levels to mutants with four different internode phenotypes (slender, tall, dwarf, nana). The quantity of polyamines measured correlated directly with the amount of internode elongation. DFMO and DFMA, when applied exogenously, inhibited both internode elongation and the increase in polyamine titer; the simultaneous application of putrescine and agmatine was able to partially restore elongation. These results were interpreted to mean that polyamines might be important in the portion of the GA response that is due to cell division, but that polyamines could not be involved in the portion of the GA response due to cell elongation.

Martin-Tanguy and colleagues have conducted extensive research into the physiology of polyamine conjugates in flowering plants.[9,14,16] In their 1979 and 1982 papers they examined the hydroxycinnamic acid amides in cytoplasmic male sterile lines of *Zea mays* (maize). This compound was found to be absent in anthers from plants with the Texas male sterile cytoplasm, and present in plants that also contained the appropriate nuclear restorer gene. Analysis of the postfertilization events of cob and grain development and maturation showed no changes in the hydroxycinnamic acid content in male sterile and male fertile lines. These changes are thus developmentally restricted just to the affected anthers.

A nonflowering line of tobacco, RMB7, was studied by Dumas and colleagues,[10,17] and compared to the standard tobacco variety, Xanthi n.c. Aromatic amine conjugates that normally increase in tobacco plants as they flower were found to be at very low levels in RMB7. Martin-Tanguy[16] has also reported interesting experiments with this RMB7 line in connection with tobacco mosaic virus infection. The virus caused necrosis over the whole RMB7 plant, whereas it is normally restricted to a local lesion response in variety Xanthi n.c., which has high levels of amide conjugates. Some caution is needed in interpreting these studies, since RMB7 is not a single gene mutation in an otherwise Xanthi n.c. genetic background. The line RMB7 originated from a hybrid of *Nicotiana tabacum* cv. Maryland mammoth with *N. rustica*; this hybrid was subsequently backcrossed to the *N. tabacum* parent seven times to generate RMB7. This method of interspecies hybridization followed by a backcross program is one of the classic methods of producing tobacco lines with altered flower development.[18] The origin of RMB7 means that it certainly has multiple genetic differences with Xanthi n.c. The differences between RMB7 and Xanthi in amide concentration, in flowering, and in viral infection could thus be the result of the multiple genetic differences, and may not be correlated with each other.

The phenolic acids that are frequently conjugated to polyamines are synthesized beginning with the enzyme phenylalanine ammonia lyase. Berlin[19] has studied a *p*-fluorophenylalanine resistant cell culture of tobacco that has high levels of cinnamoyl putrescines. The activities of OrnDC and ArgDC were increased three- to sixfold in the resistant line over wild type; labeled precursor feeding experiments demonstrated that most of the putrescine in the resistant line ended up as a conjugate, while a substantial free putrescine pool remained in wild type. Thus the activity of the conjugating enzyme may have been increased in this cell line.

Very interesting work on the role of ArgDC and putrescine during carrot somatic embryogenesis has been done in recent years. Feirer et al.[20] demonstrated that DFMA would block the transition of growth as callus to growth as embryogenesis, while it would not block growth of callus alone; putrescine would reverse the blockage. Fienberg et al.[21] examined polyamine synthesis in a carrot cell mutant that would not go through the somatic embryogenic pathway. Normally, carrot cells switch from growth as callus to embryogenic growth by removal of the plant hormone auxin from the medium. The cell line W001C has high internal levels of auxin, and thus cannot go through embryogenesis. When placed in medium without auxin, the mutant line also failed to display the increase in polyamine content that wild-type cells do. The activities of SamDC and ArgDC were shown to be suppressed by auxin, so that the ability to synthesize higher levels of polyamines seems to be a required step in this embryogenic differentiation process.

As noted early, Shevyakova et al.[11] have selected a NaC1-resistant cell line of *Nicotiana sylvestris*, and examined the polyamine metabolism in this line. Initial growth of cells in NaC1 showed an increase in spermidine and spermine levels. Longer term growth of adapted lines showed in contrast high levels of putrescine and low levels of spermidine and spermine. The levels of proline, an amino acid long associated with osmotic stress, also increased in the NaC1 treated lines.

The research on the hydroxycinnamic acid conjugates in male sterile maize on the analysis of polyamine levels in nonembryogenic carrot cultures are the best examples of this type of mutational analysis. In the case of the male sterile maize, the genetic basis of the mutation has been fully worked out. More analysis of other biochemical alterations is needed to demonstrate the importance of the hydroxycinnamic acid conjugates relative to the other biochemical and physiological alterations that occur in these lines. In the case of the nonembryogenic carrot culture, the nature of the primary biochemical alteration, the overproduction of auxin, has been well studied. A full genetic analysis is missing in this case, although since the line W001C was isolated in a single step from wild type, there is a good chance that only one genetic difference occurs between the mutant and wild type. A genetic analysis would help rule out the (unlikely) possibility that the changes in polyamine synthesis are the result of a second mutation that is genetically independent of the primary auxin-related defect.

IV. REGULATION OF POLYAMINE BIOSYNTHESIS DEDUCED FROM VARIANTS

A careful comparison of wild type and mutant plant cell cultures, when grown in media modified by the addition of polyamines, polyamine synthesis inhibitors, growth regulatory agents, or under stress conditions, should reveal much about the regulation of the pathway. In plants, putrescine is a precursor of pyrrolidine alkaloids, tropane alkaloids, and pyrrolizidine alkaloids,[22] and is found predominantly conjugated to hydroxycinnamic acid;[4,9,15,16] yet, relatively little attention as has been paid to the factors which regulate the utilization of this diamine. Recently, Tiburcio et al.[23] have shown that growth of tobacco callus on high levels of NAA results in the formation of putrescine and spermidine conjugates whereas during growth in low NAA, nornicotine and nicotine levels increased. Their results suggest that bound putrescine can act as a pool for pyrrolidine alkaloid formation and that changes in ArgDC levels correspond to changes in alkaloid levels.[24] A more direct approach to unraveling the source of putrescine destined for alkaloid production was reported by Tiburico and Galston.[24] They showed that inhibition of ArgDC effectively inhibits the biosynthesis of nicotine and nornicotine while inhibition of OrnDC was less effective. Also, the flow of 14-C from arginine into nicotine is much more efficient than that from ornithine.

A putrescine-utilizing mutant in cell culture has been described by Flores and Filner[4] which differs from the wild type in that these cells efficiently convert putrescine into GABA. In peas, putrescine can be converted into GABA by diamine oxidase and pyrroline dehydrogenase. No such enzymatic activities were found in the putrescine-utilizing mutant. The utilization of putrescine as a primary nitrogen source in the mutant affects the intracellular levels of free and bound putrescine; although conversion into GABA occurs rapidly, the possibility exists that these cells utilized cinnamoyl putrescine as a substrate for production of GABA. Interestingly, it appears that under certain conditions, the cinnamoyl-putrescine pool in these cells can also be utilized for maintaining the free putrescine pool, a capability which was not observed in wild type.

Until recently, systematic studies of the effects of inhibitors of polyamine synthesis on the utilization of polyamines in culture or of the effects of exogenous addition of polyamines have not been published. The two decarboxylases involved in putrescine production can be inhibited by DFMA (ArgDC) and DFMO (OrnDC). Whereas some plants are sensitive to DFMO, others are insensitive to this inhibitor.[25,26] In general, growth of tissue cultures of *Petunia*, *Nicotiana*, and *Vigna* was reduced by several inhibitors of polyamine biosynthesis; in some cases growth was restored by addition of putrescine or spermidine.[27] Little is known about the effects of DFMA. This may be due in part to the restricted availability of the inhibitor. However, treatment of carrot cultures with 1 m*M* DFMA resulted in a 50% reduction of embryogenesis after transfer from nonembryogenic medium into an embryogenic medium.[20] Interestingly, during carrot embryogenesis ArgDC activity was increased nearly

12-fold.[28] The inhibition by DFMA could be reversed by addition of 100 μM putrescine, spermidine, or spermine.[20]

We have observed in tobacco suspension cultures, that the inhibition of OrnDC is invariably lethal, whereas inhibition of ArgDC is without consequence on the growth rate.[8] This seems to apply only to cultures with an adequate nitrogen source which are not in process of adapting to an environmental stress. Furthermore, we have observed that addition of DFMA to cell suspensions can be lethal when ArgDC activity is elevated; this prompted us to explore the effects of the decarboxylase inhibitors as well as the effects of polyamine tite- on the decarboxylase activities in tobacco suspensions. It had previously been demonstrated that addition of sublethal levels of MGBG to tobacco cell cultures results, on SDS-PAGE, in the appearance of a predominant band of 35,000 daltons.[1] We have identified this band and have also determined the mechanism by which the induction occurs. Purification of the band in a nondenatured form was accomplished by ammonium sulfate precipitation, DEAE ion exchange chromotography, and absorption to MGBG-Sepharose. On SDS-PA gels the isolated peptide was a near-homogenous band with the same molecular weight and isoelectric pH as the MGBG-induced band. SamDC activity copurified with the band and was elevated[18] fold compared to SamDC in untreated cultures. To explore the mechanism by which MGBG treatment results in the increase in the abundance of SamDC we used a double-labeling protocol to assess, first, the relative rate of synthesis of SamDC in the presence and absence of MGBG, and second, the relative rate of turnover of the enzyme as a function of the extracellular MGBG concentration. The results of these experiments revealed that the increase in the abundance of SamDC was due entirely to stabilization of the protein; the rate of synthesis of the protein was not affected by MGBG. Interestingly, the half-life of SamDC is remarkably short and is on the order of 30 min (Figure 1). Only one other enzyme has been reported to have such a high rate of turnover; OrnDC from mouse kidney has a half-life of about 20 min.[29] The very high rate of turnover of SamDC in tobacco suggests that regulation of the enzymatic activity, and hence the titer of putrescine and polyamines may be a critical function; mobilization of transcription and translation may in some instances be too slow a process to respond to a demand for polyamine production, whereas slight changes in the rate of degradation, given a high rate of synthesis, can result in dramatic increases in enzyme abundance in a short time.

An unexpected observation from these studies was the treatment of cultures of MGBG resulted in the loss of ArgDc activity. Comparison of the time course of the loss of ArgDc activity induced by with either cycloheximide or MGBG revealed the two to be coincident (Figure 2). Using either 10 μM cycloheximide of 1 mM MGBG, the half-life of the ArgDc activity could be estimated, and in both cases was about 6 hr. This suggests that MGBG may be blocking the synthesis of ArgDc. Similar results were obtained when cultures were exposed to 1 mM spermidine, spermine, putrescine, or agmatine. Apparently ArgDc synthesis is extensively regulated by the availability of extracellular polyamines whereas OrnDc activity is relatively unaffected.

ArgDc activity in tobacco cultures is very sensitive to stress.[30] This is consistent with the observations of Galston and others that ArgDc in oat seedlings is increased dramatically by exposure to low pH, osmotic shock, and potassium starvation.[30] These treatments also result in elevated ArgDc activity in tobacco suspension cultures.[31] In addition, during the transition from callus growth on agar to liquid suspension growth, ArgDc activity is augmented.[8] We have found that during these intervals when ArgDc activity is increased, DFMA can become a lethal inhibitor. The effectiveness of DFMA in altering the growth of tobacco cells appear to depend on the severity of the stress condition as well as the extent of increase in ArgDc activity.[32]

As mentioned previously, the DFMO-resistant cell line, *Dfr1*, has very low total OrnDc activity (10%); about half of the total activity is insensitive to the inhibitor in vitro suggesting

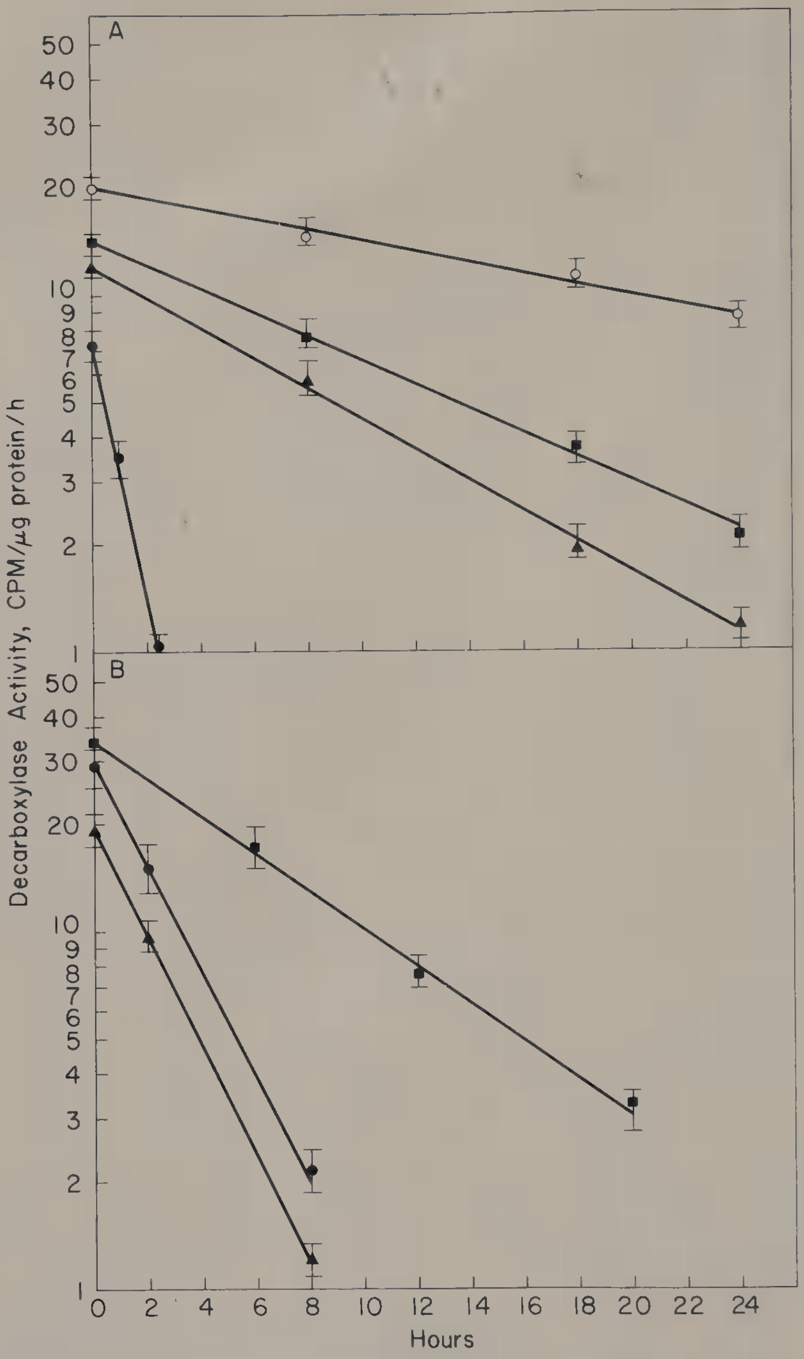

FIGURE 1. The half-lives of OrnDC, ArgDC, and SamDC arginine in tobacco cell suspension cultures. Cycloheximide was added to cultures and aliquots were removed at the indicated times to assay enzyme activity.[8] (A): SamDC activity after exposure to various concentrations of MGBG; ● no MGBG, ▲ 0.5-m*M* MGBG, ■ 1-m*M* MGBG, and ○ 5-m*M* MGBG. (B): ▲ OrnDC activity, ● OrnDC activity after exposure to 5-m*M* MGBG, ■ ArgDC activity.

that perhaps one allele of OrnDc is producing a DFMO-insensitive enzyme. This model does not account for the very low total OrnDc activity. In addition, the ArgDc activity is increased 20-fold and this has resulted in 20-fold excess of intracellular free putrescine. It appears then, from these results, that production of putrescine from arginine in *Dfr1* had overcompensated for a reduction in total decarboxylation of orinthine. In suspension cultures, *Dfr1* can readily proliferate and displays some unusual growth characteristics. We have used this culture to assess the significance, during stress, of elevated levels of free putrescine. As reported in the literature, elevated putrescine titers are a consequence of many stress

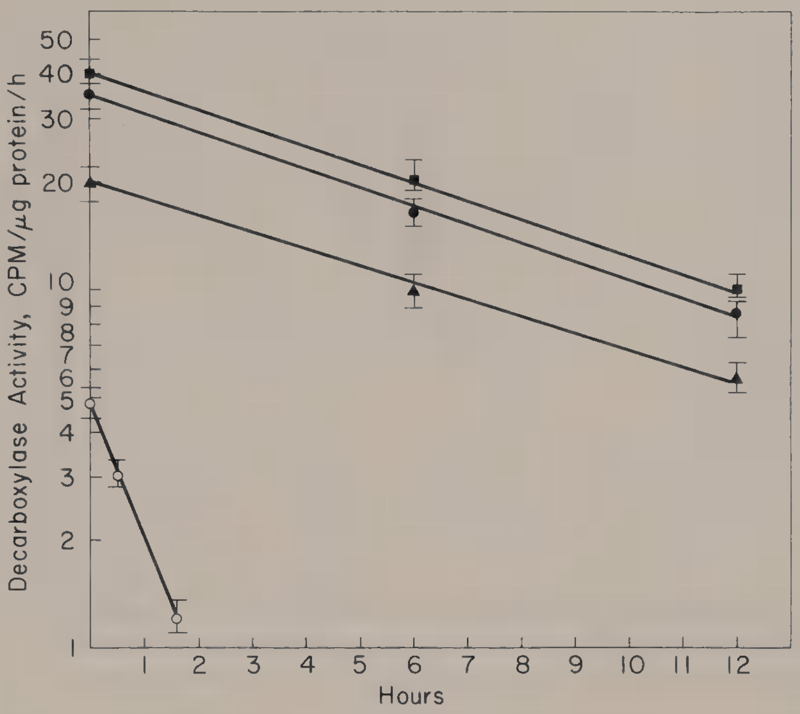

FIGURE 2. Effects of exogenous spermidine and MGBG on the half-lives of ArgDC and SamDC enzyme activities. Spermidine and MGBG were added to cultures and then aliquots were removed at the indicated times to assay enzyme activity.[8] ○ SamDC activity after exposure to 0.5 m*M* spermidine, ▲ SamDC activity after exposure to 0.5 m*M* spermidine and 0.5 m*M* MGBG, ● ArgDC activity after exposure to 0.5 m*M* MGBG, ■ ArgDC activity after exposure to 0.5 m*M* spermidine.

conditions in a variety of plants. It has never been ascertained, however, whether increased ArgDc and putrescine levels are deleterious to the well being of the plant or whether they in some way enhance the ability of the plant to survive a stressful environment. Compared to wild-type cultures, *Dfr1* is relatively insensitive to low pH stress in that resumption of a normal rate of growth after low pH shock is achieved at a significantly higher, faster rate than wild type. In some conditions, *Dfr1* cells survive acidic conditions which are lethal to wild-type cultures. We have been exploring the mechanism by which *Dfr1* is capable of growth after severe stress and have found that these cells secrete, in excess, free putrescine. We do not yet know if this is the only basic compound secreted by *Dfr1*, nor do we know if this accounts for the neutralization. Putrescine secretion by *Dfr1* cells upon a challenge by acidic conditions can reach a level of about 10 μ*M* in the extracellular medium. Addition of DFMA to the medium at the same time as the acidic challenge however does not result in a slowed rate of acid neutralization. In fact, wild-type cells are capable of neutralizing the extracellular medium at the same rate as *Dfr1* cells, suggesting that putrescine secretion is not a significant contributer to the ability of cells to buffer their environment. Why then should an increased synthesis of putrescine specifically from arginine be an important response to stress? Are there differences in the utilization of this putrescine under different stress conditions.? It has been suggested that synthesis of putrescine is a response to acidification of the cytoplasm and ultimately helps to regulate cellular pH. The fact that spermidine or spermine levels are not generally effected by stress suggests that the utilization of free putrescine into pathways other than the production of polyamines may be an important response. Understanding the factors which dictate that putrescine be utilized as a basic metabolite or as a precursor of secondary metabolism will be an important goal in the next few years; the putrescine-utilizing line of Flores and Filner[4] is an important first step in that regard.

V. CONCLUSIONS

As expressed earlier, a mutational analysis is an enormously powerful tool to help sort out the difference between correlations and causation. In order to meet the requirements of a proper mutational analysis, great care is needed to demonstrate that the genetic lines being compared differ only in a single locus, and that the biochemical consequences of this alteration are well worked out. Probably none of the papers reviewed here properly meets these criteria. Certainly our own work on directly isolating mutants in the polyamine synthesis pathway has insufficient genetic and biochemical analysis to have proven that the changes in polyamine synthesis we observe are directly correlated with the developmental aberrations we see in regenerated plants.

In spite of these drawbacks, however, there is beginning to be enough cumulative evidence from several groups to draw some conclusions. There is evidence from several systems that polyamines are involved in cell division rates, and in some developmental processes — notably embryogenesis and floral morphogenesis. It is reasonable to argue tht the involvement in cell division rates and the cell cycle is the primary event through which polyamine metabolism is exerting its effects. Plant development differs from animal development in the presence of a rigid cell wall around the protoplasm. The consequence of this is that there are no morphogenetic movements of plant cells during normal development; cell/cell contact in plants is primarily restricted to a given cell and its clonal relatives. To a large extent then, plant development is controlled by varying rates and patterns of cell division, coupled with subsequent cell elongation. The influence of polyamines on plant development may thus be through variations on the theme of control of the cell division cycle. A prediction of this sort of model is that cell division cycle mutants produced through other methods, through analysis of other cell-cycle-tied biochemical pathways should have some of the same phenotypes as do the polyamine lesions.

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Section C.
Polyamines and Diseases

Chapter 11

POLYAMINES IN HEALTH AND DISEASE

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TABLE OF CONTENTS

I. Introduction 164

II. Normal Events 164

III. Growth Disorders 164

IV. Genetic and Chromosomal Diseases 165

V. Polyamine Excess: The Hyperpolyaminemia States 167
  A. Toxicity 167
  B. Clinical Conditions 170
    1. Mesangial Expansion 170
    2. Liver Disorders 171
    3. Myocardiopathy 171
    4. Adult Respiratory Distress Syndrome 171
    5. Acute Renal Failure 172
    6. Toxemia 172
    7. Dysimmunity 173
    8. Transplantation 174
    9. Coma 174

VI. Miscellaneous Disorders 174

Acknowledgments 176

References 176

I. INTRODUCTION

This brief commentary is for clinician and basic scientist. In vivo and in vitro phenomena resulting from polyamine (PA) — spermine (SPM), spermidine (SPD), putrescine (PTC), and cadaverine (CDR) — interactions with macromolecules and cell systems will be scrutinized here. Relevant clusters of available information from the fields of pathophysiology, pathological biochemistry, and histopathology have been drawn together. While primary emphasis will be devoted to circumstances of known PA body fluid change and associated tissue pathology, highlights of some new and important findings about normal PA-related life processes will also be described.

II. NORMAL EVENTS

In the complex interplay between mother and infant, it would appear that the regulation of PA synthesis in the lactating breast may be a function not only of maternal nutrition,[1] but also of the infant's tongue strokes to the mother's nipples.[2] The considerable quantities of PAs found in mother's milk may have a role in stimulating the growth of various lactobacilli, the protective bacterial flora of the newborn gut.[3,4] In addition, recent studies of the newborn have shown a correlation between PA metabolism in the brain,[5] and interruption of normal maternal-infant interaction, specifically tactile stimulation. In preweanling rat pups separated from their mothers, tissue ornithine decarboxylase (ODC) was significantly decreased. Serum growth hormone (GH) levels and the sensitivity of tissues to exogenously administered GH were also decreased. These observations were not related to changes in nutrition, changes in environmental temperature, nor the action of adrenal stress hormones. GH levels rose to normal in sensory-deprived rat pups with initiation of brush stroking to imitate maternal licking patterns, but not by kinesthetic or vestibular stimulation. Similar GH changes were observed in preterm human neonates with body stroking. A 47% increase in growth, measured by weight gain, was observed in stroked vs. nonstroked preterm human neonates, without group differences in caloric intake.[6] This PA research strongly reinforces the view that it is vital to maintain sensory stimulation in order to promote normal somatic growth as well as intellectual development in infants.

Since circadian changes in hormone levels are routinely found in body fluids, PA changes are to be expected. Changes in the sum of PA synthetic and degradative activities are reflected in fractional urine collections during 24-hr periods. PA-related information on these and other rhythms such as menstrual, cellular, and reproductive cycles is available.[7-10]

Normal tissue levels of SPD and SPM are commonly in the 1 to 2 μmol/g wet weight range.[11] By contrast, normal plasma and serum PA levels are in the high picomole-low nanomole per milliliter range.[12] When plasma and RBC-PA levels were determined from single samples and compared, RBC-PA levels were one to two orders of magnitude higher. Thus, differences in compartments may vary as much as one to three orders of magnitude. Normal body fluid values have been determined.[3,12-16] It has been reported that in man there is a significant decline in circulating and excreted PAs with age.[17]

III. GROWTH DISORDERS

With the discovery that PTC was an absolute requirement for growth of *Hemophilus parainfluenza*, a role for PAs in growth processes was established.[18] Hypophysectomy reduced SPD concentrations in rat liver and GH administration restored levels to normal.[19] The role of ODC in these responses was then identified.[20,21] Dwarfed children with isolated GH deficiency responded to hormone repletion by correcting low plasma and urine-PA excretion rates toward normal.[17] Discrimination between GH-deficiency states and end-organ failure, as in Turner's syndrome, was accomplished by administration of exogenous GH and concurrent measurement of blood PAs.[17,22] A related condition, acromegaly, a pituitary disorder involving the excessive release of GH, has been reported to have increased urinary excretion of PAs.[23] Beckwith-Wiedemann syndrome (BWS), a congenital growth disorder, was found to have essentially nondetectable 24-hr SPD and SPM excretion rates[24] (see Section IV).

Intrauterine growth retardation (IUGR) becomes apparent in the last trimester when the placenta is aging and is under metabolic stress. While due to a variety of causes, IUGR frequently occurs with progressive vascular insufficiency of the placental unit. In IUGR serial determinations of maternal whole blood, PA levels do not rise appropriately in the third trimester.[25,26]

Disturbances in PA metabolism may also result from abnormal diet. Alcohol metabolites appear to perturb PA metabolism and function. One third of infants born to alcoholic mothers have fetal alcohol syndrome (FAS). Key features include IUGR, microcephaly, maxillary hypoplasia, reduced width to palpebral fissures, and cardiac abnormalities.[27] FAS infants have developmental delay and mental retardation. In rabbits given isolated hepatic alcohol infusions, conformational changes in polyribosomes occurred and albumin synthesis declined.[28] The effect was overcome by the additional infusion of SPM or arginine.[29] This reversibility suggests the accumulating alcohol metabolite acetaldehyde might be binding free SPD and SPM via a Schiff-base mechanism, thus reducing the stability of polyribosomes and adversely affecting protein synthesis. This functional depletion of PAs occurring at critical times during embryogenesis (before day 56) would be reflected in organ anomalies, tissue hypoplasia, and dysmorphia. A recent study indicated prophylactic aspirin, a transplacental agent, significantly reduced FAS-like limb-bud abnormalities in alcohol-treated mice.[30]

Thalidomide, a sedative-hypnotic drug, was the source of an epidemic of human fetal limb-bud malformations.[15] While a specific role for PAs in the disordered embryogenesis has not been assigned, it is of note that PAs, in particular SPD, form complexes with thalidomide through spontaneous acylation.[31]

IV. GENETIC AND CHROMOSOMAL DISEASES

PA disturbances have now been described in a number of heritable disorders, such as psoriasis, BWS, disordered amino acid transport, ornithine transcarbamylase deficiency, and sickle cell disease.

Psoriasis, a disease of abnormal epidermal proliferation, has been found to have increased local ODC activity, elevated systemic PAs, and increased urinary PA excretion.[32] PA synthesis inhibitors such as difluoromethylornithine (DFMO) now appear to be successful in curbing ODC activity when applied with a lipophilic carrier.[33] There is an equilibrium between HLA types and psoriasis, suggesting a genetic origin, the mode of inheritance being dominant.[32] Recent gene-mapping studies involving the etiopathogenesis of psoriasis localize the defect to chromosome 6 in the regions of the C and D loci of the major histocompatibility complex.[34]

BWS, a rare congenital disorder of infancy, presents with macroglossia, somatic gigantism, and visceromegaly.[35] Patients afflicted with BWS were found to have 24-hr urine excretion of PTC four times the mean normal values. SPD and SPM were, for all practical purposes, undetectable.[24] Multifactorial inheritance is suggested. BWS is ordinarily sporadic but some familial cases indicating autosomal dominant inheritance have been reported. With the advent of new banding techniques, partial duplications of chromosome llp have been identified in some BWS patients as well as others with only limited expression of the disorder.[36]

Heritable disorders of amino acid transport may also have disturbed body fluid PAs.

Lowe's syndrome, Fanconi's syndrome, as well as cystinuria may have grossly elevated SPD and PTC excretion in the urine. This appears to be due to disordered renal tubular transport mechanism for dibasic amino acids and it is consistent with known normal inward transport of SPM and SPD by proximal renal tubular epithelial cells.[37]

An ornithine transcarbamylase deficiency (OTCD) patient showed urinary SPD levels to be a full order of magnitude above normal.[37] This observation may provide a critical clue to the devastating toxicity seen in OTCD. In this X-linked dominant disease males usually die in the neonatal period. The disease in heterozygotes is expressed as lethargy, irritability, growth failure, protein avoidance, developmental delay, vomiting, behavior disorders, convulsions, coma, and death.[38] During acute episodes OTCD shares many clinical symptoms, signs, and biochemical abnormalities with Reye's syndrome, a known hyperpolyaminemic state.[39] In its more chronic form, its expression is much like chronic renal insufficiency in the infant and small child.[40]

With the report of a five- to tenfold elevation of PAs in sickle cell blood, it is evident that homozygous sickle cell disease (SSD) patients suffer from chronic hyperpolyaminemia (HPA) and, based on several considerations, a significant hyperpolyaminuria.[41] The membranes of these actively hemolyzing cells contain higher concentrations of PAs, particularly SPM, than normal cells.[42] Moreover, PA-rich immature RBCs are typically present in large numbers due to chronic bone marrow stimulation, secondary to decreased life span of sickle cells.[43] In addition, predictable i.v. plasma PA excesses are to be expected during sickle cell crises when peripheral tissue ischemia and microinfarction, autosplenectomy, and renal injury occur.

While some of the data is conflicting, there is strong evidence supporting the view that abnormalities of PA metabolism are operating in the hereditary muscular dystrophies. Erythrocyte SPM levels were reported both elevated and normal in patients with Duchenne muscular dystrophy (DMD).[44,45] Both increased and normal urinary excretion of PAs were also reported in DMD.[45,46] Analysis of DMD muscle samples revealed markedly elevated concentrations of PTC and SPD, but SPM content was found to be normal.[47] In myotonic muscular dystrophy (MMD), basal serum and urine levels of SPM and CDR were elevated. SPD levels were depressed. Following human growth hormone (hGH) administration to MMD patients, serum and urine PTC, SPM, and CDR increased but SPD remained unchanged. In limb girdle dystrophy (LGD) patients' basal serum and urine SPD, SPM, and CDR were elevated. Urinary excretion of PTC was increased but serum levels were normal. Administration of hGH significantly increased serum and urine PTC, SPD, SPM, and CDR. Both MMD and LGD showed anabolic responses to hGH administration. DMD did not.[48] Normal controls showed neither the hGH anabolic responses nor the subsequent increases in serum or urinary PAs. Thus, these three dystrophic muscle disorders revealed abnormal but somewhat different expressions of PAs dysmetabolism.[46] Increases in skeletal muscle PA concentrations observed in dystrophic patients suggests muscle as the likely source of elevated PAs in serum and urine. Since elevated PA concentrations have been found in muscle of DMD, MMD, LGD, and in dystrophic mice and hamsters it is possible an unknown mechanism, reflected in the observed hGH hypersensitivity, permits PAs to rise to intracellular levels observed in these conditions. It is well known that the range of intracellular SPD concentrations for proper messenger reading and optimal protein synthesis is narrow. In vitro PAs in excess lose their growth-promoting properties, inhibit cells, and may be cytotoxic. Finally, where measured, the presence of increased CDR in serum, urine, and tissues reflects catabolism of lysine. Loss of this essential amino acid in significant amounts could further contribute to muscle wasting. Exploring the PA biosynthetic and degradation pathways in the muscular dystrophies would appear to be promising. This is particularly so when pathway inhibitors are now readily available for laboratory and clinical investigations.

Table 1
CLINICAL PERTURBATIONS IN PA METABOLISM

Etiology	Altered state	Ref.
Increased production	Pregnancy	129
	Fetus and newborn	26
	Acromegaly	23
	Malignancies	49
	Psoriasis	32
Decreased biodegradation	Liver insufficiency	51, 113
Retention	Renal insufficiency	50, 107
Enzyme dysfunction	OTC deficiency	37
Mixed etiologies	Burns	56
	Cytotoxic drugs	52
	Exercise	125
	Infections: bacterial and viral	23, 49, 113
	Radiation	53
	Rheumatoid arthritis	49
	Systemic lupus erythematosus	57
	Transplant rejection	147
	Trauma/surgery	55
Altered renal transport	Cystinuria, Fanconi's syndrome Lowe's syndrome	37, 59
Impaired production	Intrauterine growth retardation	25, 26
	Hormone dependent dwarfism	17
	Beckwith-Wiedemann	24
PA antagonism (suspected)	Fetal alcohol syndrome	29
	Thalidomide	31

V. POLYAMINE EXCESS: THE HYPERPOLYAMINEMIA STATES

A. Toxicity

HPA is commonly seen in circumstances of increased production, retention, and failure of biodegradation. Blood PA elevations in pregnancy and malignancy reflect active cell proliferation.[26,49] Elevated levels are seen in CRF and advanced liver disease.[50,51] When tissue damage is induced by a surgical trauma, irradiation, UV light, burns, cytotoxic drugs, or viral or bacterial infections, nonspecific elevations of PAs have also been observed.[52-56] Similar changes have been demonstrated in immunoinflammatory diseases such as systemic lupus erythematosus, rheumatoid arthritis, and polymyositis.[23,57,49] HPA is ordinarily associated with a demonstrable increase in urinary PA excretion. However, uremia appears to be an exception.[58] In genetic disorders of renal tubular transport where large quantities of PAs have been documented in urine,[37] no systematic examination of changes in other body fluids has been undertaken. However, a preliminary study on a small number of patients with Lowe's syndrome reported some serum-PA elevation.[59] Table 1 reveals the scope of clinical perturbations in PA metabolism.

''Autointoxication'' as a term has been out of favor with clinicians for many years. However, uremic toxins, the toxic ''X'' factors of cancer, toxemia of pregnancy, and toxic encephalopathies are still clinically valid terms serving as shorthand for poorly defined but obvious stereotypic clinical entities. Moreover, there still remains a need to enter ''plasma factors'' into pathogenic schemes of individual clinical disorders such as acute renal failure (ARF), adult respiratory distress syndrome (ARDS), and shock heart to explain the natural history of such problems. Based on known adverse effects of PAs on biological systems, organ histology, and responses to therapeutic interventions, most of the above poorly defined humoral substances appear to behave like PAs in excess. We have coined the general term

''PA stress'' to encompass a broad range of changes manifest in both animals and man under circumstances of HPA.

Uremia serves as a clinical HPA example of how pervasive toxic changes may be. As to presentation, much depends on the degree and duration of uremia. Anorexia is common.[50] Nausea and vomiting may be striking. Peripheral neuropathy, altered temperature regulation, convulsions, and coma are frequent neurological complications.[60] Nonspecific anemia and depressed cellular immune mechanisms are consistent features.[61,62] Disturbances in electrolyte, fat, and carbohydrate metabolism and elevations of blood histamine, catecholamines, and prolactin have been reported.[63-66] Perturbations consonant with many of the above changes have been produced by PAs both in vivo or in vitro in experimental settings.[50,67-70] Moreover, other clinical HPA conditions share a number of the above features with uremia.[50,61]

PA toxicity probably results from a number of factors. The combined characteristics of low molecular weights (88 to 202 daltons), aqueous solubility, and polycationic charge due to nearly complete protonation at biological pHs are unique in nature. The remarkable interactive and regulatory properties of PAs include ionic bonding, cation exchange, chelation, Schiff-base reactions, conjugation, coacervation of large molecules, and liberation of biologically active degradation products such as ammonia, hydrogen peroxide, aldehydes, and acrolein upon oxidation. PAs have been shown under in vitro conditions to inhibit such vital enzyme functions as Na^+-K^+ ATPase and phospholipase activities and to interfere with mitochondrial transport and oxidative functions.[71-73] The acute systemic toxicity of PAs appears to be a function of charge. Parenteral SPM is much more toxic than SPD and the toxicity of diamines PTC and CDR is negligible.[74] Intraperitoneal administration of SPM has been shown to be diffusely toxic, producing pathological changes in visceral organs.[75] Preincubation of PAs with an amine oxidase or parenteral injection of the enzyme prior to SPM decreased acute toxicity.

In vitro hydrogen peroxide stimulates release of histamine from mast cells.[76] PA injections cause a rise of histamine in the blood and tissues of experimental animals.[77,78] The toxic effects of PAs are ameliorated by administration of H1 blocking agents.[78,79] Marked increased diamine and PA oxidase activities have been reported in sustained HPA conditions such as pregnancy and uremia.[80,81] In the presence of amine oxidase activity, PAs inhibit mitogenically stimulated lymphocytes.[82] Under similar circumstances neutrophil motility is inhibited. The rapidly occurring nontoxic and reversible suppression of activity may be caused by aldehydes. By contrast, acrolein, a very slowly elaborated breakdown product of PA-derived aminoaldehydes, has been shown to be cytotoxic and causes irreversible inhibition of cell proliferation. Acrolein also inhibits neutrophil locomotion.[83,84] However, a role for acrolein in the above responses is considered much less likely than the rapidly produced and very reactive aminoaldehydes. Neutrophils become immobilized in the capillaries of the lung following i.p. injection of PAs and then undergo cytolysis.[75] PAs inhibit RBC production both in vivo and in vitro, and this can occur in vitro in the absence of diamine oxidase (DAO).[85,86] Finally, PAs administered parenterally to experimental animals have the property of accumulating in tissues.[87,88] Since the efficiency and fidelity of protein synthetic machinery depends on rigorous maintenance of narrow intracellular PA concentrations, such accumulations may be factors in PA cytotoxicity.

The morphological expressions of experimental PA stress vary depending on the daily parenteral dose of PAs, duration of treatment, and the particular organ involved. Parenteral SPM is cytotoxic to proximal convoluted tubule cells of the kidneys.[89,90] Based on renal arterial infusion studies, these effects appear to be direct.[91] Type I pneumocytes are susceptible in vivo.[69] The nephrotoxic threshold in mice for i.p. SPM was approximately 10 to 15 mg/kg of body weight in single daily doses. Toxic synergy appeared with combined SPM and SPD injections.[69] Cytotoxicity was seen at 24 to 48 hr in the proximal convoluted

Table 2
RECENTLY DEFINED EFFECTS OF EXOGENOUSLY APPLIED POLYAMINES 75, 92, 93, 167

- Pathophysiological alterations
  - Renal corticomedullary blood redistribution
  - Glomerular capillary dilation
  - Cardiac capillary engorgement (RBCs)
  - Pulmonary
    - Neutrophil endocapillary stasis
    - Endolymphatic edema
    - Macrophage migration

- Proproliferation
  - Epicardial mast cells
  - Pulmonary
    - Fibroblasts
    - Type II pneumocytes
  - Glomerulomegally with mesangial cell hyperplasia

- New Product Formation
  - Mesangial matrix

- Abnormal deposits
  - Hepatic microvesicular neutral fat
  - Glomerular fibrin-like extracellular deposits
  - Renal epithelial and endothelial myeloid bodies, intra- and extracellular
  - Intracellular cardiac calcium
  - Intra- and extracellular fluid vacuoles in various tissues

- Cytotoxicity
  - Proximal tubule epithelium
  - Type I pneumocyte
  - Cardiac myocytes (subsarcolemma)
  - Pulmonary alveolar hemorrhage (injury site unknown)

- Heterogeneity
  - PA-affected tissues have piebald pathological changes

tubules and cardiac myocytes following daily i.p. injections of SPM 15 mg/kg and SPD 30 mg/kg of body weight. Type I pneumocytes showed susceptibility. Juxtaposed and morphologically similar cells show different responses, i.e., one undergoing necrosis and its neighbor showing little or no visible change. This heterogeneity of cell damage was quite characteristic of PA intoxication. Proliferation of glomerular cells and type II pneumocytes was observed in animals whose proximal convoluted tubules appeared normal following 21 daily injections of SPM 15 mg/kg body weight.[69,92] PAs have also been reported to promote in vitro proliferation of fibroblasts and arterial smooth muscle cells.[93,94] In addition to cytotoxicity and proliferation, evidence for coacervation of extracellular proteins was noted. Precipitation of Tamm-Horsfall (TH) glycoprotein in distal convoluted tubules and collecting ducts and granular fibrinogen-like deposits in the glomeruli of PA-treated rats and mice were identified. In vitro TH protein readily precipitated in the presence of SPM.[95] In Table 2 are listed five groups of changes observed by light and electron microscopy in the hearts, lungs, livers, and kidneys of experimental animals following parenteral PAs. These are discussed under organs involved in clinical disorders.

HPA is also associated with mediator and hormone release. Sharp increases in plasma prolactin is seen in Reye's syndrome and in toxemia of pregnancy (PGTX).[96,97] In toxemic women near term, prolactin levels were twice normal. Severity of disease in Reye's syndrome correlates with plasma prolactin levels. Elevated prolactin has also been reported in trauma, hepatic, and acute renal failures.[98-100] Histamine injections in animals stimulate a rise in plasma prolactin.[101] As noted before, PAs stimulate a rise in plasma histamine. Increased in vivo adrenergic activity follows the injection of SPM. This is abolished by adrenal demedullation or reserpine administration.[102] Rapid redistribution of renal corticomedullary blood flow also occurs following injection of SPM.[95]

Vitamin metabolism may also be perturbed. HPA patients tend also to have vitamin B_6 insufficiency.[68] In vitro studies revealed rapid pyridoxal phosphate-PA complex formation.[103] The complexes were also found in human urine.[104] Many disturbances seen in HPA states have been produced in experimental vitamin B_6 insufficiency. These included microvesicular fatty liver, cellular dysimmunity, peripheral neuropathy, and seizures.[105,106]

B. Clinical Conditions

As can be discerned from Table 1, hyperpolyaminemic states are general and nonspecific. In the following section, discussions of both organ damage as a cause of HPA and the effects of HPA on organ morphology and function will be presented.

In uremia, PAs accumulate in blood indicating the importance of the kidney in PA homeostasis (see Section A). Elevated concentrations have been found in serum, RBCs and tissues.[50,58,107,108] Despite elevated blood-PA levels, 24-hr urine PA excretion was reduced in ambulatory uremic patients compared to normals. A severalfold increase in SPD, much of it conjugated, has been observed in serum of uremic patients.[109] While some decline in PA serum levels occurs following hemodialysis of uremic patients, levels do not return to the normal range.[50]

The anemia of uremia, a nonspecific normocytic normochromic anemia, is thought to result from altered erythropoietic activity, toxic inhibition of the bone marrow, and shortened RBC survival time.[105] There is an inverse correlation between serum-SPM levels and hematocrits in uremic patients.[107] SPM or SPD, when added to erythropoietin-stimulated cultures of fetal mouse liver or normal human bone marrow inhibited erythroid colony formation in a dose-dependent manner.[86] Inhibition caused by uremic serum and active fractions was abolished by the addition of specific anti-PA antibodies. Further, studies using fetal mouse liver cell cultures proved SPM and SPD, of a variety of amines tested, to be among the most potent inhibitors of 59Fe incorporation into newly synthesized heme.[111] In the intact rat, continuous delivery of low-dose SPM produced a hypoproliferative anemia without concurrent azotemia.[85] Finally, it should be noted that the anemia of uremia is remarkably similar in kind, if not degree, to the nonspecific anemia commonly seen in cancer patients, an HPA state with relatively normal renal function.[50]

1. Mesangial Expansion

One of the perplexing problems of renal insufficiency is the tendency of remaining functional kidney tissue to deteriorate. This process continues long after the original compromising insult has passed. The period until uremic symptoms supervene may be a matter of years. The process of deterioration can be reproduced in healthy experimental animals by surgical reduction of renal mass. Dietary protein restriction may slow the process in patients and experimental animals. In the postsurgical remnant kidney of experimental animals there is increased renal blood flow, glomerular hyperfiltration, and proteinuria. Glomerular tuft enlargement is the result of cell proliferation. The mesangial cells in particular continue to proliferate and lay down increased amounts of extracellular matrix. With time this mesangial expansion crowds the capillaries and results in ischemia, sclerosis, and glomerular obliteration.[112] Either dialytic maintenance or transplantation are required, or death ensues. The glomeruli of mice treated with SPM in low doses for 3 weeks were analyzed morphometrically.[92] Glomerular enlargement and proliferation of the three major glomerular cell types was noted. However, measurements revealed disproportionate increase in the mesangium, the mesangial cells plus matrix. There was a smaller but significant increase in capillary area. These changes were consistent with the early finding in the renal reduction model. These responses of mesangial cells may be similar to stimulatory effects of PAs on arterial smooth muscle cells. Both uremic serum and PAs induce these cells to proliferate.[93] This is not surprising since mesangial cells are believed to be modified smooth muscle cells. Because uremia is an HPA state, the clinical and experimental deterioration of renal function may be, in large measure, the result of proliferative glomerular responses to PAs. Other HPA states such as idiopathic nephrotic syndrome, cirrhosis, and sickle cell disease may develop similar mesangial changes.

2. Liver Disorders

In viewing disorders of the liver, several aspects of PA metabolism require consideration. On the one hand, acute liver cell injury, as in infectious hepatitis, would be expected to release PAs into the circulation. On the other hand, chronic parenchymal damage reducing effective liver mass as seen in alcoholic cirrhosis would be expected to seriously compromise biodegradation of PAs elaborated from peripheral tissues. The relative contributions of each of these mechanisms to HPA in liver disease and the promotion of progressive autotoxic hepatic damage remains to be determined. Studies have been carried out to determine the effect of hepatic malignancy, hepatitis, and cirrhosis on body fluid PA levels.[51,113] Levels as high as 5 to 30 times normal have been reported in liver failure. Elevations of PTC and SPD are most commonly observed. These findings demonstrated the liver, like the kidney, plays an important role in PA homeostasis.

While possible hepatotoxic effects of elevated i.v. PAs in these conditions are unknown, the administration of the parenteral SPM and SPD to mice induce dramatic changes in hepatocyte morphology over a period of days. A microvesicular steatosis of the type seen in Reye's syndrome and Sheehan's fatty liver of pregnancy is observed.[114,115]

3. Myocardiopathy

PA toxicity to the heart also deserves consideration. Myofibrillar degeneration, also called selective myocardial necrosis, is a diffuse, patchy, essentially noninflammatory lytic disorder of the myocardium. It has been described in uremia, shock, Reye's syndrome, pheochromocytoma, postcardiac surgery, and unexplained sudden cardiac death.[116,117] The lesions are limited to individual and small groups of myocytes unrelated in distribution to coronary blood vessels. Clumping, disorganization, and proteolysis of cardiac myofibrillae and Z bands occurs early. Segmentation of the muscle fibers with banding, subsarcolemmal edema, and dislocation of mitochondria are typical features. Increased calcium has been identified in the lesions. Animals injected with daily doses of PAs develop ventricular lesions in 24 to 48 hr. These continue to progress, plateauing after 7 to 10 days.[69,118] Intracellular calcium is observed. PA changes are, for all practical purposes, identical with those seen clinically. From a functional point of view, it should be noted that well-described but incompletely isolated humoral depressor factors are known to adversely effect cardiac function in critically ill patients and animals in shock.[119] That PAs are depressor factors seems likely. This is supported by recent observations of direct adverse effect of PAs on in vitro cardiac muscle function.[120]

4. Adult Respiratory Distress Syndrome

While there are many reported events precipitating the adult respiratory distress syndrome (ARDS), tissue injury by physical, chemical, or septic means appears to be the underlying causes. Humoral induction of ARDS is suggested by the frequency of inciting injury remote from the lung.[121] Early ARDS, which usually begins to develop in a matter of hours, is marked by increased pulmonary capillary permeability, migration of neutrophils into the capillary beds, and the release of hydrolytic enzymes.[122] Intraalveolar microhemorrhages, microatelectasis, and sloughing of type I pneumocytes follows. Because of increasing interstitial edema the lung gradually stiffens. Over a period of several days proliferation of type II pneumocytes and fibroblasts begin the process of obliteration of alveolar spaces. In experiments with mice the morphological effects of daily i.p. injections of SPM or SPM plus SPD in the status of the lung were determined. Endocapillary neutrophil leukostasis was particularly prominent and was recognizable 30 min after SPM injection. Progressive lung changes over a period of 7 days were entirely consistent with the evolution of the clinical syndrome. After 21 days, type II pneumocyte and fibroblast proliferation resulted in the progressive collapse of the alveolar air sacs.

Considerable indirect evidence suggests PA oxidation may play a role in PA-induced ARDS. First the cells of the alveolar air sac receive direct and continued exposure to oxygen. PAs accumulate in lung tissue.[88] PAs present in lung tissue may serve as a permeability factor whose activity can be inhibited by an H_1 blocker.[78] Peroxisomes, organelles containing PA oxidase, are present in type II cells. The oxidation of PAs involves the liberation of H_2O_2.[123] H_2O_2 induces the release of histamine from mast cells.[76] Finally, the transtracheal administration of histamine sets up local proliferative reactions in lung parenchyma similar to ARDS.[124] The polycationic aminoglycoside antibiotics which cotransport with the PAs but are not biotransformed in the body produce ARF in a fashion morphologically similar to the PAs.[90] These antibiotics, however, do not cause clinical or experimental ARDS.

5. *Acute Renal Failure*

The common form, a nonspecific acute renal failure (ARF), occurs in settings of extensive tissue damage and, thus, it is not unusual for the patient to have ARDS. While the circumstances precipitating ARF are many, it is typically seen in crush injury, severe burns, sepsis, i.v. hemolysis, and following administration of polyaminated drugs such as the aminoglycosides. The highest serum levels of PAs observed in our laboratory were from a child in renal shutdown from the hemolytic uremic syndrome. It is not uncommon for children with Reye's Syndrome and patients with Sheehan's fatty liver of pregnancy to develop ARF. Severe exercise has also been noted to increase PA blood levels.[125] It is generally viewed that hypovolemia, decreased renal perfusion, and the release of tissue substances from remote sites of injury may be major early determinates.[126] An initiating hyperadrenergic corticomedullary redistribution of blood, varying degrees of proximal and distal tubule damage, and nephron obstruction in the distal convoluted tubules by proximal tubular detritis in a matrix of TH protein are thought to be important events.[127]

In the experimental setting, high doses of parenteral SPM induce changes much like those in clinical nonspecific ARF. Redistribution of renal blood flow is rapid, proximal tubular damage and TH cast formation are again outstanding features. TH protein rapidly forms a rubbery precipitate in the presence of SPM.[90,95] Since PAs are naturally occurring substances accumulating in the extracellular space with tissue injury, the excess excretory load places the proximal tubules in jeopardy. The stress of marathon running is seen to precipitate acute renal failure.[128] Severe exercise caused SPD to rise as much as two and one half times above control levels. The resultant HPA and hypovolemia under circumstances of relative renal hypoxia, would appear to set the stage for compromised renal function. Any significant degree of rhabdomyolysis with release of PAs and myoglobin would have a further destabilizing effect on tubular function and TH protein.

6. *Toxemia*

In pregnancy a profound and yet physiological HPA state exists.[129,26] Low early (10 to 16 weeks) and high late peaks (34 to 38 weeks) are observed for blood SPD and SPM. Such elevated SPM levels as seen in the last trimester do not appear in normal processes elsewhere in nature. The first peak is associated in time with morning sickness and the second with the occurrence of Sheehan's fatty liver of pregnancy and toxemia (preeclampsia). Fetal tissues, the placenta, and amniotic fluids are rich in PAs. An enzymatic barrier, containing diamine and PA-oxidizing enzymes, is strategically placed within the maternal decidual element.[123,130] This may serve not only to reduce the maternal ''amine load'', but by virtue of inhibitory properties of PA, oxidative products may prevent maternal cellular immune mechanisms from setting up hostile reactions against fetal tissue antigens.[131] Increased plasma enzyme activity is seen with progression of gestation.

Toxemia is heralded by hypertension, proteinuria, and edema at about the 28th week of pregnancy. At risk are healthy primigravidas and women with multiple pregnancies (twins and triplets).[132] Increased maternal risk also occurs when underlying vascular disease resulting from diabetes mellitus, essential hypertension, or chronic renal disease is present. It is of particular interest that classical symptoms and signs of toxemia can develop well before the 20th week in thwarted pregnancy if rapidly proliferating residual intrauterine trophoblastic tissue remains. In this setting, plasma amine oxidase activities are low.[133]

In toxemia a cluster of diagnostic changes occur in the kidney. These can be readily distinguished from those due to underlying glomerular disease or essential hypertension by renal biopsy. By light microscopy enlarged and ''anemic'' glomeruli are seen.[134] Capillary spaces appear partially obliterated with what appears to be vacuolated cell detritus. The glomeruli may fluoresce strongly to tagged antifibrinogen antibodies.[135] Proximal tubules show hyaline droplet formation. By transmission electron microscopy these capillaries appear obstructed by what most authors attribute to swollen and vacuolated endothelial cells. However, a significant part of the endocapillary change could be due to impaction of upstream cellular debris at ultrafiltration sites. Mesangial cell proliferation with expansion of matrix and interposition were present. Cell vacuolization and myeloid body accumulations may be striking. Interspersed finely granular fibrinogen-like precipitates in the subendothelial and the mesangial cleft regions are typical features. Foot process fusion was rare. Similar changes, including proteinuria, have been produced in nonpregnant female rats following injection of daily parenteral SPM.[69] However, at the levels used to induce the glomerular lesions, proximal tubular damage occurred. It should be pointed out that parenteral injections of endotoxin, a potent stimulus to tissue ODC activity, produce glomerular lesions said to be indistinguishable from those of toxemia.[137]

Based on present knowledge, it seems reasonable to suggest toxemia results from placental PA overflow at a time when the accretion of fetal mass is rapidly approaching a physiological limit. A state of relative PA overproduction, i.e., in the case of twins, triplets, or somatomegalic infants of diabetic mothers may exist. A second consideration is inadequate delivery of oxygen to the placental oxidizing enzymes at a time when maternal SPM is at its highest. This may result from the relatively immature uterine vasculature development in primigravidas in as much as toxemia rarely occurs with a second pregnancy. Further, uterine arterial pathology is present in both essential hypertension and diabetes mellitus. Placental infarctions, acute atherosis with decreased luminal size of decidual arteries, senile trophoblastic changes, and decreased maternal blood flow through the placenta are found in toxemia.[138-141]

7. Dysimmunity

Disturbances in the cellular immune system are commonly observed in HPA states. Depressed lymphocyte responses to mitogenic stimulation and blunted or absent cutaneous delayed hypersensitivity are seen in pregnancy, uremia, chronic liver disease, cancer, and sickle cell disease.[142-146] Depressed mitogenic responses were elicited when the subject lymphocytes or normal-test lymphocytes were incubated with the subject's own plasma.

This indicated a humoral factor-inhibiting transformation. Experimentally, it has been shown that in order for SPM and SPD to inhibit mitogenically induced blast transformation of murine lymphocytes, fetal calf serum must be present.[82] Fetal calf serum contains DAO. Increased activity of this enzyme has been reported in pregnancy, uremia, hepatic disease, and some cancer patients.

8. *Transplantation*

Longitudinal monitoring of changes in serum SPM in recently hemodialyzed renal allograft recipients was carried out to determine if a rise in SPM might presage early rejection prior to onset of ordinary clinical indices.[147] Following renal transplantation, previously elevated SPM values rapidly returned to the normal range. In all cases, SPM levels then increased and exceeded 2 SDs above normal mean level early in rejection. The varying degrees of transient mild renal insufficiency associated with these episodes failed to explain the disproportionate SPM rise with rejection. While SPM levels did not appear to be significantly influenced by surgery, graft irradiation, or pharmacological agents, an ongoing oscillatory pattern of SPM serum levels was observed in all patients. An exaggerated ''hectic'' spiking pattern was observed as rejection progressed. More recently, serial urinary PA analysis in heart transplant patients confirmed the value of PA monitoring in diagnosing rejection.[148] Total urinary PAs increased 1 to 3 days before a proven rejection in conventionally treated patients. In cyclosporine-treated patients urinary acetylputrescine to N1-acetylspermidine ratio became elevated before rejection.

It is useful to point out that DFMO significantly prolongs skin allografts in mice differing at the H-2 major histocompatibility locus. This model provided a major test of immunosuppressive capability.[149] Various regimens combining DFMO and (methylglyoxal-bis-guanylhydrazone) MGBG enhanced graft prolongation. The use of MGBG was not without systemic toxicity.

9. *Coma*

There is a strong association between HPA states and coma, i.e., uremia, liver failure, Reye's Syndrome, Sheehan's fatty liver of pregnancy, and the eclamptic phase of toxemia. In uremia, restoration of most CNS functions can be completely achieved by hemodialysis and in hepatic coma by hemoperfusion.[150,151] However, very little is presently known to document direct or indirect toxic effects of extracellular space accumulation of PAs on brain metabolism. It is known that in vitro oxygen consumption by rat cortical tissue is inhibited by SPM.[152]

Finally, it is not uncommon for more than one visceral organ to fail with severe trauma, septic states, or chemical insults. Increasing attention is being given to as yet to be defined humoral substances adversely affecting the host in what has come to be known as multiple organ systems failure.

VI. MISCELLANEOUS DISORDERS

Periodontal disease — Periodontal disease induced in dogs by subgingival ligatures caused a sharp rise in ODC activity of the adjacent tissue, but no noticeable increase in local PA levels.[153] When ligatures were removed, a sharp rise in tissue PTC level and a parallel rise in ODC activity occurred. Applications of PTC to periodontal pockets seemed to enhance the rate of healing.[153]

Deafness — Deafness is seen in uremia, but renal transplantation reverses the defect.[154] It has been proposed that uremic toxins might produce cochlear hair cell lesions leading to hearing loss. The irreversible cytotoxic effect of many aminated ototoxic drugs is enhanced by the concurrent administration of loop-inhibiting diuretics such as ethacrynic acid.[155] In a recent study, SPM was administered to guinea pigs followed in 2 hr by this loop diuretic.

A loss of high-frequency auditory function was observed 2 days later. However, no change in either electrophysiological AC cochlear potential or N_1 thresholds was observed. It appeared that observed changes in thresholds were the result of central nervous system changes and not the cochlea.[156] The findings are in accord with the reversible hearing loss of uremia. Moreover, hair cells contain microbody-like structures (presumed peroxisomes) which, in the liver at least, oxidize PAs.[157]

Neuropsychiatric — Mental aberrations are frequently encountered in patients whose disease processes cause HPA. Changes range from simple obtundation and deficient cognition to florid affect alterations and schizoid symptoms. Systemic lupus erythematosus, uremia, cancer, eclampsia, the postpartum period and Reye's syndrome all have such derangements. Support for a possible PA role in precipitating psychiatric disorders has been strengthened by recent attention focused on therapeutic agents containing a SPD moiety whose side effects include depression or psychosis.[158] These include the antimalarials chloroquine, mepacrine, and amodiaquine. Intraventricular injection of PAs in rats produced insomnia and abolish feeding.[159]

Drug Addiction — In vitro PA metabolism in morphine-binding neuroblastoma X-glioma hybrid cells was profoundly effected by acute and chronic administration of opiates and during drug withdrawal. Acute inhibition of adenylate cyclase activity by morphine was followed by a compensatory enzyme increase during chronic incubation, the cells becoming increasingly tolerant, and thus, addicted.[160] Acute administration of morphine and other opiates decreased cAMP concentrations. cAMP-dependent protein kinase and ODC activities and ribosomal RNA synthesis were all decreased.[161] A corresponding decline in intracellular SPD and PTC was observed. Chronic exposure of the hybrid cells lead to morphine tolerance. Little reduction of cAMP level or depression of enzyme activities was noted. When opiates were withdrawn and the antagonist naloxone added, cAMP levels and enzyme activities increased 100%. The behavior of ODC in drug-accommodated cells subjected to withdrawal raised the question of whether a surge in PA synthesis might be responsible for seizures seen in clinical withdrawal. Seizures are common in HPA states and PAs induce seizures in animals.[162]

Prematurity — In contrast to IUGR, prematures (<37 weeks) are small but appropriate for dates. However, IUGR infants may also be premature. Preterm blood PA concentrations were reported to be consistently higher than those in full-term infants. The greater the degree of prematurity, the higher the PA levels at birth.[26] Prolonged PA elevations were observed with increasing degrees of prematurity.

Vomiting — SPM injected intramuscularly into humans caused vomiting and proteinuria.[67] Nausea and vomiting are common characteristics of HPA states such as early pregnancy, Reye's syndrome, Sheehan's fatty liver of pregnancy, advanced uremia, and oncolytic and irradiation therapies. Since histamine is a known emetic in both dogs and humans, the liberation of histamine may explain the vomiting associated with HPA.[163,164] Thus, it is reasonable to propose an indirect mechanism for PA activation of the chemoreceptor trigger zone. Supporting the view is the well-known utility of H_1 blocking agents in ameliorating clinical nausea and vomiting of central origin.

Therapy — Promising improvements in clinical therapeutics have been suggested by trials being carried out in the treatment of sleeping sickness (*African trypanosomiasis*) and pneumonias due to *Pneumocystis carinii*. A recent report indicates that 96 of 103 late-stage sleeping sickness cases appeared to be cured by the administration of DFMO.[165] A combination of the cytostatic action of DFMO on the trypanosome and the competency of the host's humoral immune mechanisms are thought to result in a successful outcome. DFMO has also been prescribed in the successful treatment of pentamidine resistant *P. carinii* pulmonary infections. Control of this typically fatal infection, commonly seen in patients with cancer, immunosuppressive therapy, and AIDS, with a relatively nontoxic agent will find great utility.[166]

In summary, a group of interesting and important PA-related conditions have been brought together here. Included are normal life processes, genetic disorders, intoxications, life-threatening diseases, and innovative therapies. Certain untouched areas such as cancer are discussed elsewhere in the text. The increasing momentum of basic science information generation in the PA discipline may lead clinicians to regard PAs as a Rosetta Stone for medicine.

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Chapter 12

POLYAMINE METABOLISM AND NEOPLASTIC GROWTH: A PROGRAMMED DEREGULATION?*

Giuseppe Scalabrino and Maria Ferioli

...hypotheses non fingo. Quicquid enim ex phaenomenis non deducitur, ''hypothesis'' vocanda est; et hypotheses seu metaphysicae seu physicae, seu qualitatum occultarum, seu mechanicae, in ''philosophia experimentali'' locum non habent. In hac philosophia propositiones deducuntur ex phenomenis et redduntur generales per inductionem.

I. NEWTON, Philosophiae Naturalis Principia Mathematica, Vol. III, Part II, Scholium Generale.

L'inconnu de la vie des êtres est comme celui de la nature, que chaque découverte scientifique ne fait que reculer mais n'annulle pas.

M. PROUST, A la recherche du temps perdu, La prisonnière.

TABLE OF CONTENTS

I. Introduction 184

II. Polyamine Metabolism in Experimental Oncology 186
  A. Two-Stage Carcinogenesis in Mouse Skin 186
  B. Carcinogenesis in Rodent Liver 193

III. Polyamine Metabolism in Cultured Mammalian Neoplastic Cell Lines 197
  A. Different Hepatoma Lines 197
  B. Different Nerve Tumor Cell Lines 198
  C. Mouse Lymphoma and Leukemic Cell Lines 200
  D. Ornithine Decarboxylase and Polyamine Levels in Cultured Mammalian Neoplastic Cells During Differentiation Induced by Different Chemicals 200

IV. Polyamines in Human Oncology 201

V. Polyamine Metabolism in Carcinogenesis Induced by Physical Agents 203
  A. Carcinogenesis Induced by UVB Irradiation 203
  B. Carcinogenesis Induced by Ionizing Radiation 203

VI. Epilogue 203
  A. The Programmed Deregulation of Polyamine Metabolism in Neoplastic Cells 204
  B. Autocrine Regulation of Poliferation of the Neoplastic Cell: A Role for Polyamines? 205

References 205

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[*] This chapter is dedicated to our master and mentor, Prof. E. Ciaranfi, on the occasion of his 80th birthday (April 17, 1986). His valuable teaching and his farsighted advice in the early phases of our scientific development have had an enduring influence on us.

I. INTRODUCTION

At the present time there are two main lines of investigation into the biology of cancer. The first is essentially genetic, concerning chromosomal aberrations and oncogenes in cancer cells. The second is essentially biochemical in the broad sense of the word, concerning energy metabolism, membrane structure, antigenic composition, synthesis of cyclic nucleotides, and prostaglandins in cancer cells. It is almost redundant to say that both approaches are equally important and complement each other, and both can be viewed as Ariadne's threads for unraveling the oncological labyrinth. This is to say that neoplasms, both human and experimental, in spite of their heterogeneity, need a genoma that is not necessarily unique or valid for all tumors, and consequently, that neoplasms can also have different gene expressions, related to the type of tumor and its phase of growth. It is now clear that having the gene modifications is not of itself a sufficient condition for malignant transformation, but that a given biologic context is required before they become operative. What is important is to understand that the biology of cancer is programmed, albeit polymorphic in its phenotypic expression. Weber called this concept ''reprogramming'' of gene expression in neoplasia and, sometimes, ''the biochemical strategy'' of cancer cells.[1-4] The true situation is, therefore, what we might call, by an oxymoron, an ''order of the disorder'' of neoplastic growth.

Furthermore, it is well known that the biochemical strategy of and the logic of gene expression in cancer cells include, and are therefore mirrored by, both quantitative and qualitative abnormalities.[1-4] However, a merely quantitative approach seems to have had its day, because of the erratic results, and it did not serve as a gateway to understanding the essence of the biochemical strategy of neoplastic cells. The qualitative approach is much more promising. It has revealed chiefly the dearth or even the absence of some control mechanisms in the biochemistry of cancer cells. This type of deregulation of cancer cells is of selective and energetic advantage for uncurbed and autonomous growth, which is widely considered to be the hallmark of neoplastic growth. Just as there are these qualitative differences in neoplasms, there are also quantitative ones. Again, with this type of study it was possible to identify shared enzymic and metabolic deprogramming in the various neoplasms, beyond the sheer complexity and apparent diversity. In other words, a segment of gene expression that is essential for neoplasm has been identified and the biochemical pattern that characterizes the committment of cancer cells to indefinite replication has been detected.[1-13]

Generally speaking, the phenomenon of growth in eukaryotic cells is essentially a phenomenon of energy transformation. In the normal quiescent cell there is a nearly complete balance between synthetic and breakdown processes, which means that the fuel molecules that enter the cells are employed essentially to maintain the metabolic steady state and to renew cellular structures. Instead, in growing cells, a part which is quantitatively variable from time to time of fuel molecules is transformed into new protoplasm, which means that much metabolic energy is employed to synthesize macromolecules and other biomolecules. However, few would contest the statement that the division and differentiation of somatic cells in higher organisms are regulated by multiple controls. In the last decades it has become even more evident that the control of normal growth is not entirely dependent on the sheer availability of metabolic energy to the cells, but is based on other physiological regulatory molecules with different structures and produced by different cell types. All these molecules are able either to stimulate or to inhibit or in some other way to condition cell growth. Among these growth-controlling substances, it seems to us appropriate to include the large family of various growth and maturation factors, chalones, cyclic nucleotides, prostaglandins, and, of course, polyamines. The physiological growth-promoting substances can be locally acting substances, stimulating growth of the same cells by which they are produced, or remote-acting substances, stimulating cell growth far from their site of production. Obviously, polyamines are growth-promoting, locally acting substances, when considering, as in this present chapter, only the effect of polyamines on cell growth, leaving out their other, different biological effects.

It is clearly outside the limits of this chapter to present a comprehensive summary of all the different aspects and, even more, to discuss analytically all the different aspects of the topic ''polyamines and neoplastic growth.'' Our aim is to review selectively and in detail the literature on the connections between polyamines and cancer in mammals that has been published since the 1980s. The literature for this topic that appeared before 1980 has already been thoroughly discussed in our previous two-part review.[14,15] The reader will find there the appropriate references and an in-depth discussion about those. This chapter is an updating of our previous review. At this time we wish only to recall some classical facts and some basic beliefs about the connections between polyamine metabolism and the neoplastic disease process. We think that a reader who is less well acquainted with this topic may benefit from these introductory remarks, which serve as fundamental starting points for understanding the more recent advances in the field.

1. Investigation of the links between polyamines and tumors can be divided historically into two facets.[14] In the first of these, quantitative differences between the polyamine levels of neoplasms and those of the normal corresponding organs were found. In the second, which began more recently, qualitative differences in the regulation of polyamine biosynthesis between normal tissues and neoplasms (isozymes of L-ornithine decarboxylase (EC 4.1.1.17), ODC), loss of the ODC circadian rhythm, decreased responsiveness of ODC to putrescine inhibition, and imbalance in ornithine metabolism have been found.[14]
2. The quantitative differences in polyamine biosynthesis in neoplastic tissues indicate that polyamines are important tools, but by no means the only tools available to neoplastic cells for uncontrolled growth.
3. The qualitative differences in polyamine biosynthesis regulation in neoplastic tissues indicate that deregulation is one of the ways but by no means the only one, by which neoplastic cells escape the normal controls of growth.
4. The fact that different types of growth stimuli, some carcinogenic and some not, increase polyamine biosynthesis in the target organ is only an apparent evidence for the casual involvement of polyamines in neoplastic transformation. This belief is not valid, because it is actually firmly established that the various carcinogens apparently trigger the display of some biochemical program, which has been identified in different species and in tumors of different cell origin, but which, above all, is independent of the carcinogen.[9,12,16,17] Since there is constant involvement of polyamine biosynthesis when cell growth is stimulated by whatsoever stimulus, the increase in ODC activity in the target tissue is considered to be a fundamental part of the so-called pleiotypic program set off by a growth-promoting stimulus.[18] In other words, the increase in polyamine biosynthesis is tightly connected with growth processes, not for neoplastic growth only.[14,15]
5. It is becoming increasingly apparent that cancer development in some of the mammalian organs or tissues is a stepwise process involving alterations of cell populations through many steps (paradigmatic to this regard are the two-stage models for the carcinogenesis of skin and liver in rodents).[19-25] Furthermore, the clonal evolution of tumor cell populations proceeds through the selection of variant subpopulations from a common progenitor.[20,22,23] Polyamine biosynthesis, noticeably the step of ODC, is involved in and linked to both the initiation process and the promotion process.[14,15] Studies of experimental hepatomas with different growth rates, which means studies with different

tumors of the same histogenetic origin but with different cell populations, and of long-term chemical hepatocarcinogenesis, which means a model for hepatocarcinogenesis in which successive populations of cells sequentially emerge, have demonstrated that the increase in hepatic ODC activity is linked not only to the existence of neoplastic transformation, but also to the degree of malignancy of the neoplasm and to the stage of the neoplasm, i.e., to the neoplastic progression.[5,6,14,26] This result has been also confirmed in some human primary tumors, such as skin epitheliomas and tumors of central nervous system.[27,28]

6. Although neoplasm is a pathologic condition definable only in vivo in the intact animal or human being, the involvement of polyamine biosynthesis has also always been demonstrated in different in vitro models used for investigating malignant transformation of eukaryotic cells by different agents, including viruses.[14,15] And this is of particular interest, because the malignant transformation is not an all-or-none phenomenon, but has various degrees and steps.
7. As normal eukaryotic cells become senescent, i.e., when there is slowing of their rate of division and cessation of growth, the biosynthesis of polyamines progressively and significantly decreases.[29] Contrariwise, embryonic and fetal tissues, which are in active and rapid proliferation, have strikingly elevated polyamine biosynthesis.[30]
8. Another important finding stressing the absolute requirement of mammalian cells for polyamines for continued proliferation was obtained in recent years. The genetic approach, based on isolation of mutants with altered polyamine metabolism regulation and expression, and formerly limited to prokaryotes and lower eukaryotes only, has now been extended to mammalian cells. Mutants of CHO cells, which are polyamine deficient or with severely reduced ODC activity, have been isolated and studied.[31,32] Additional evidence that mammalian cells must have putrescine for survival and replication, was obtained with these CHO mutants.[31,32]

On the basis of these few basic concepts, most of which have been discussed in the above-mentioned review, we can now discuss in detail most of the recent information obtained in the last 5 years about the connections between polyamines and neoplastic growth in both human and experimental oncology.[14,15]

II. POLYAMINE METABOLISM IN EXPERIMENTAL ONCOLOGY

A. Two-Stage Carcinogenesis in Mouse Skin

In recent years, studies of polyamine biosynthesis have been based on the two-stage carcinogenesis protocol of mouse skin, with greater understanding as this model of carcinogenesis has been thoroughly investigated in different strains of mice and in cultured epidermal cells.[33-35] It has been suggested that the tumor promotion event can be divided into two stages, the first of which has been brought about paradigmatically by limited application of 12-*O*-tetradecanoylphorbol 13-acetate (TPA), whereas the second can be brought about paradigmatically by repeated applications of mezerein (MZ), a diterpene similar to TPA in its biochemical and morphological effects, but a weak complete promoter.[36-40] The first (I) stage of promotion is called conversion and the second (II) stage of promotion is called propagation.[41] Combined treatment after tumor initiation with 7,12-dimethylbenz(a)anthracene (DMBA) gives a tumor response similar to single-stage or complete promotion by TPA alone.[42] This division of the promotion stage is further supported by the identification of selective inhibitors for one or another stage of promotion. For example, tosylphenylalanyl chloromethylketone (TPCK) specifically inhibits stage I promotion but not stage II, whereas retinoic acid (RA) inhibits stage II but not stage I.[38,42,43] The percentage of the mice with skin tumors is reduced by one half by the combination of either of the two treatments [DMBA + (TPA + TPCK) + MZ] or [DMBA + TPA + (MZ + RA)].[42] However, it should be noted that TPCK was found to have only a slight inhibitory effect on TPA-induced epidermal ODC activity, thus raising once again the much-debated question as to whether epidermal ODC changes are or are not a key factor in the promotion stage of the two-stage skin carcinogenesis model.[38]

The phorbol ester tumor promoters have been shown to have a wide range of biological effects on eukaryotic cells and can exert their highly pleiotropic effects on the growth, function, and differentiation of a large variety of cell lines.[44-47] Certainly, most of our information about the mechanisms of action of this class of agents is derived from studies of the potent skin-tumor promoter TPA. At the biochemical level, it appears that the major difference between initiators and the phorbol ester tumor promoters is that whereas initiators (or their metabolites) bind covalently to cellular DNA, the primary site of action of the phorbol ester tumor promoters is the cell membrane, following receptor binding.[46,48,49] For instance, a prolonged receptor occupancy by the promoter appears to be necessary in HeLa cells for the promoter induction of ODC activity.[50] Several results demonstrate (1) the existence of specific high-affinity, saturable ''phorboid receptors'' in membrane preparations and intact cells from various animal and human tissues and (2) the heterogeneity (or subclasses) of receptors for phorbol esters and related tumor promoters.[45,51,52]

However, a crucial question for understanding the biological basis of the effects induced by phorbol esters is whether tumor promoters act entirely via inductive or hormone-like or growth factor-like effects, thus mediating clonal expansion of previously mutated cells, or whether they can themselves produce stable and heritable changes in the cell phenotype. It is largely accepted that in contrast to oncogenic viruses, chemical carcinogens and tumor promoters cannot induce new genetic information into cells. Nevertheless, tumor promoters are potent and reversible inhibitors of a variety of programs of cell differentiation, and they can also be potent inducers of other differentiating programs.[44,45,47,53] Furthermore, although most of the pleiotropic effects of tumor promoters in cell cultures are dependent on the continuous presence of the promoter (*vide supra*[50]), there are a few examples in which tumor promoters induce irreversible effects.[51,52] This is not surprising, since the prevailing view of today is that tumor promotion has at least two likely targets within the cell and therefore involves two probably independent mechanisms: (1) one at the regulator gene in the nucleus and (2) one at the cell surface membrane.[54]

The morphological effects of TPA are pertinent to the above question, since it has been shown that TPA induces the appearance of dark basal keratinocytes, the so called ''dark cells.''[42,55] MZ, on the contrary, shows very little ability to induce these dark cells, whereas ethyl phenylpropiolate (EPP), a hyperplaseogenic agent without promoting activity, does not induce any dark cells.[56] Thus, the induction of these dark cells seems to be connected with stage I promotion by TPA. These dark cells may be stem cells, which are normally present in large amounts in embryonic and newborn skin, and also in papillomas and carcinomas of the skin, but only in very small quantities in adult skin.[37,42,55] Several investigators have demonstrated that the basal cells rather than the differentiating cells of the epidermis are the target cells for ODC induction by TPA in vitro.[57-59]

There was much more ODC induction by TPA in mouse epidermal cells grown in medium with a low calcium concentration (which technique selects for basal cell growth) than in cells grown in medium with normal calcium concentration.[57-59] Furthermore, there was a rapid loss of responsiveness to TPA after exposure of cells grown in low calcium medium to a medium with normal calcium concentration, which parallels the onset of well-defined differentiating morphological changes.[57-59]

The specificity of this phenomenon and the noninvolvement of medium calcium concentration in causing this phenomenon is demonstrated by the fact that the well-known inducibility of ODC by UV light in the same cultured mouse epidermal cells is not at all affected by the change of calcium concentration in the medium.[57] Nevertheless, the importance of intracellular calcium in epidermal ODC induction by TPA was demonstrated by the prevention or at least by the strong reduction of the enzyme induction in adult skin explants by chelation of extracellular calcium with ethyleneglycolbis-(β)-aminoethyl ether) *N,N* tetraacetic acid (EGTA) or by inhibiting calmodulin with trifluoroperzine.[60]

During these last years, some additional details about the biochemical events elicited by phorbol ester tumor promoters have been found.

1. The kinetics of the induction of polyamine biosynthetic decarboxylases in hairless mouse skin induced to proliferate by TPA and the kinetics of the subsequent accumulation of putrescine, spermidine (SPD), and spermine (SP) have been carefully investigated, showing a strong interdependence of these events (especially the SPD/SP ratio) with the rate of DNA synthesis.[61]
2. When the effects of a single application of TPA, MZ, or EPP on DNA synthesis and polyamine levels in hairless mouse epidermis were compared, TPA and MZ induced typical biphasic increases in DNA synthesis, putrescine levels, and SPD/SP ratio, whereas EPP induced single-peaked increases in the same parameters.[62]
3. When TPA was administered in a twofold application to mouse skin, the interval time was shown to be critical in determining the induction of polyamine biosynthetic decarboxylase levels and polyamine accumulation.[63] When the time interval between the two applications was shorter than 24 hr, the first application of TPA paradoxically induced a refractory state, because the second one was without effect on polyamine biosynthesis, whereas when the interval was longer than 48 hr, the effect of the second TPA application on polyamine biosynthesis was much greater than that observed after the first application.[63]
4. Interestingly enough, it has been demonstrated that the newborn mouse epidermis is completely resistant to TPA as hyperplasiogenic agent and as an ODC inductor agent until the 5th day after birth.[64] Starting on that day, TPA-responsiveness develops gradually and concomitantly with the morphological and biochemical conversion from the neonatal into the adult phenotype of the epidermal cells.[64] When stage I of promotion was carried out on neonatal initiated mouse epidermis during the period of TPA-unresponsiveness, there was almost no tumor development.[64] Similarly, TPA treatment was shown to be ineffective in inducing ODC activity or in enhancing DNA and RNA synthesis in rat embryonic visceral yolk sac, confirming the unresponsiveness of immature mammalian tissues to TPA.[65]
5. It was shown that TPA induces ODC activity in human skin in a manner similar to that observed in mouse skin, and that the main biochemical events triggered by TPA in mouse skin were observed in human skin samples obtained from volunteers, although there is one negative report.[66-68]
6. The cascade of biochemical events after TPA administration to mouse skin has been carefully investigated and some of these viewed as possible mediators for polyamine biosynthesis stimulation. A single topical application of TPA induced rapid and transient increases in the levels of both cAMP and cGMP in mouse epidermis, followed by the induction of ODC activity.[69] A single topical application of 3-isobutyl-1-methylxantine (IBMX), a well-known inhibitor of phosphodiesterase, greatly raised the levels of cyclic nucleotides but, surprisingly, increased the magnitude of ODC induction by TPA.[69] This last result supports the notion that TPA can also induce ODC activity by a mechanism independent of the cyclic nucleotides in mouse epidermis. In line with this are the results showing that TPA treatment was able to induce ODC activity even in some CHO cell mutants deficient in the enzyme cAMP-dependent

protein kinase, although this induction was quantitatively smaller than that observed in the parental or wild-type cell line.[70,71] Furthermore, a topical application of IBMX before TPA promotion greatly reduced the number of papillomas per mouse.[72] However, the same treatment either before or after initiation with DMBA had no effect on the development of the tumors.[72]

7. A group of studies have clearly demonstrated the involvement of prostaglandin E_2 in TPA-induced ODC activity in mouse skin and in a murine epidermal cell line.[73,74] In addition to the cyclooxygenase product (i.e., PGE_2), the lipoxygenase products and the stimulation of phospholipase A_2 activity were shown to be involved in the mechanism(s) of ODC induction by TPA in mouse skin. A variety of inhibitors of cyclooxygenase (e.g., indomethacin) or of lipoxygenase (e.g., nordihydroguaiaretic acid) or of phospholipase A_2 (e.g., mepacrine) were able to prevent the in vivo induction of ODC activity in epidermal cells by phorbol ester tumor promoters.[75-78] However, the lipoxygenase inhibitors did not inhibit the TPA-induced epidermal DNA synthesis.[78] Amazingly enough, pretreatment of cultured 3T3 cells with indomethacin was not able to prevent the TPA-induced ODC increase, although it brought about a complete inhibition of TPA-induced release of PGE_2.[79,80]
8. It has been demonstrated both in vivo (in mouse epidermis) and in vitro (in cultured mouse dermal fibroblasts) that the induction of aryl hydrocarbon hydroxylase (EC 1.14.14.2) (AHH) (a cytochrome P-450-dependent microsomal monoxygenase complex responsible for the metabolic conversion of polycyclic aromatic hydrocarbons to their active, carcinogenic derivatives) can occur without precedent or concomitant induction of ODC activity.[81,82] DMBA or 3-methylcholanthrene (MC) induced AHH activity without affecting ODC activity and, conversely, RA markedly inhibited ODC activity without affecting AHH activity level.[81,82] This dissociation between ODC induction and the AHH induction was also observed in HeLa cells treated with benz(a)anthracene, which induced AHH activity but not ODC activity.[83] All these results are quite opposite to those previously obtained by other authors (reviewed in Reference 14) that showed a close relationship between the inducibility of those two enzyme activities in inbred AHH-responsive and AHH-nonresponsive mice.
9. Equally interestingly, it was demonstrated both in vivo (in mouse epidermis) and in vitro (in isolated mouse epidermal cells) that when several different amino acids, such as cysteine, tryptophan, methionine, phenylalanine, glycine, asparagine, glutamic acid, leucine, and arginine were given before TPA, they greatly reduced the TPA-induced epidermal ODC activity, without however altering the time course for ODC induction by TPA.[84,85] This inhibitory effect of exogenous amino acids on the induction of epidermal ODC activity has also been shown when the stimulating agents were hormones, growth factors, or MZ, instead of TPA.[85] Although the mechanism for this inhibition remains to be determined, all these results are compatible with the hypothesis that the presence of an altered protein spectrum in the epidermis following amino acid administration or addition to the medium may affect the process of ODC induction by all the growth-promoting substances in the broadest sense of the word, including of course TPA.
10. This kind of research into amino acid-mediated inhibition of TPA-induced ODC activity has opened new perspectives for clarifying some other molecular mechanisms of ODC induction in skin tumor promotion process. The constituent amino acids of reduced glutathione (GSH) (γ-glutamylcysteinglycine), i.e., glutamic acid, cysteine, and glycine, and GSH itself strongly inhibited TPA-induced ODC activity in mouse epidermis both in vivo and in vitro.[86,87]

The biological importance of these observations was further substantiated by the finding that the magnitude of the inhibitory effects of cysteine and GSH on ODC induction by TPA was proportional to their abilities to decrease the incidence of skin tumors in the initiation-promotion protocol.[86] These inhibitory effects of GSH and its single constituent amino acids on both ODC induction and on the incidence of tumor development must be connected somehow to the increase in the intracellular ratio of GSH/GSSG which is caused by these substances and which effectively counteracts the decrease in the same ratio caused by TPA.[86] The modulation of the GSH/GSSG ratio might, therefore, play a key role both in tumor promotion and in ODC induction. This is in keeping with the well-known ability of tumor promoter phorbol esters to stimulate the cellular ''oxidative burst'', leading to the generation of superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH·), and to selectively inhibit epidermal superoxide dismutase (SOD) and catalase (CAT) activities, the major detoxifying enzymes for O_2^- radicals.[86,87]

Parenthetically, the production of all these oxygen metabolites has been considered as one basis for the indirect cytotoxic properties of TPA in some mammalian cells.[88] CAT, Cu-3,5-diisopropylsalicylic acid (a copper complex with SOD-mimetic action), mannitol (a scavenger of hydroxyl radicals), and D-α-tocopherol (a scavenger of free radicals) also prevented ODC induction by tumor-promoting phorbol esters in mouse epidermal cells, both in vivo and in vitro, and in a cell line of mouse mammary tumor.[77,86,89,90] Interestingly enough, D-α-tocopherol is inhibitory on TPA-induced ODC activity in mouse epidermis in vivo and in vitro, without directly modifying the intracellular GSH/GSSG ratio and without inhibiting TPA-enhanced DNA synthesis.[77,86] All these results taken together are considerable and compelling evidence for the participation of active oxygen radicals in carcinogenesis and tumor promotion by TPA as well as in ODC induction by TPA in epidermal cells.

In recent years several cell lines in culture have been identified that have different degrees of nonresponsiveness to TPA and other phorbol esters than the parental cell populations from which they were derived. While different in many respects, these variant cell lines share at least two common features: they have apparently normal specific phorbol ester binding and the molecular defect(s) that confers nonresponsiveness to phorbol esters is (are) unknown. TPA was unable to induce any stimulation of ODC activity in some mitogen-specific nonproliferative variants of 3T3 cells.[91] In a mutant 3T3 cell line defective in ion transport, ODC induction did not occur after TPA treatment, although it occurred after exposure to other growth stimuli, such as the addition of fresh medium containing serum.[92] All these results with these defective cell lines strongly support the existence of distinct, immediate postreceptor events that are initiated by various phorbol esters in the responsive cells and that trigger changes in membrane function and gene expression, leading to DNA synthesis and cell growth and/or to cell differentiation.[46,48] Refractoriness to TPA has also been demonstrated in vivo in mice resistant to urethane-induced lung carcinogenesis, in which the administration of TPA failed to induce lung ODC activity, whereas it was effective in mice responsive to urethane-induced lung carcinogenesis.[93] Therefore, all these in vivo and in vitro experiments demonstrate that the ODC-stimulating effect of TPA in various mouse strains and in various cultured cell lines also depends on genetic features of the target cells.

The crucial point of the presence of an abnormal ODC phenotype in neoplastic cells has been further emphasized by results from some authors. O'Brien's group[94,95] demonstrated that in transformed hamster epidermal cells TPA and other phorbol ester tumor promoters act synergistically with serum growth factors to induce ODC activity to a level much greater than the sum of the induction caused by each treatment alone.[94,95] This synergistic induction of ODC activity by TPA and serum is accompanied by an increased amount of enzyme protein and by an accumulation of ODC mRNAs.[95] This type of abnormality in ODC induction is not an utterly new finding, since an analogous result has been previously reported again by O'Brien's group (reviewed in Reference 14). However, O'Brien and co-workers[94] suggest that this synergistic induction of ODC activity might be characteristic of transformed cells, because it does not occur in normal epidermal or hamster fibroblast cells. Nevertheless, synergistic increases in ODC activity in bovine lymphocytes after combined exposure to phytohemagglutinin (PHA) and TPA has been reported.[96] In keeping with this, tumor-promoting phorbol derivatives were shown to enhance mitogenesis in concanavalin A (con A)- or PHA-activated murine lymphocytes, which means that tumor-promoting phorbol esters have a comitogenic activity.[97,98] Again, synergistic induction of ODC activity after the addition of TPA plus fresh medium was observed in cultured hamster tracheal epithelial cells, in which this combined treatment was followed by a wave of mitosis.[99] Therefore, although these different experiments were carried out with different experimental cell systems, i.e., hamster cells and lymphocytes, one can reasonably conclude that the synergistic induction of ODC activity seems to be characteristic of and coupled to cell mitosis per se, whether normal or neoplastic, rather than specifically related to a transformed state of the cell. To date, the only exception known is the result of Moriarity et al.,[100] who showed that the effect of the simultaneous presence of TPA and EGF on ODC activity of cultured human hepatoma cells was additive, although TPA did not inhibit EGF binding.

Interesting is the finding of Astrup and Boutwell[101] that the high levels of ODC activity in squamous papillomas of rat skin induced by the two-stage carcinogenesis protocol become established, remaining high in spite of cycloheximide treatment, which practically abolished protein synthesis. This is additional evidence, besides those summarized in Section I, for a derangement of the control of polyamine biosynthesis in tumors and possibly whenever cells are dividing.

Because the induction of epidermal ODC activity is a typical and prominent biochemical event elicited by phorbol ester tumor promoters, other compounds were screened as possible tumor promoters by testing their abilities to induce ODC activity in mouse skin. At least three classes of chemicals were identified as new tumor promoters: (1) indole alkaloids, among which the most representative substance is teleocidin;[102-104] (2) the polyacetate group, among which the most representative substance is aplysiatoxin;[104] and (3) anthracene-derived substances, among which the most representative substance is chrysarobin.[105] Furthermore, a group of compounds already known as tumor promoters, i.e., the ingenol esters, have been shown to induce epidermal ODC activity in the mouse.[106] Teleocidin induces epidermal ODC activity through a mechanism similar to that of TPA, which includes stimulation of phospholipase A_2 and both the types of arachidonate metabolites, i.e., cyclooxygenase products and lypoxygenase products.[107]

Finally, using some inhibitors helped to clarify the role of ODC in both initiation and promotion. 7,8-Benzoflavone, which inhibits DMBA metabolism, inhibits ODC induction and the formation of skin tumors by DMBA when it is applied in conjunction with this carcinogen, but failed to inhibit the TPA-induced ODC activity in mouse skin.[108,109] RA has the opposite effect, since it does not inhibit DMBA-induced epidermal ODC activity, while it is widely known to be a potent inhibitor of epidermal TPA-induced ODC activity without, however, influencing the hyperplastic response to phorbol ester tumor promoters[109] (for review, see Reference 15). These results clearly demonstrated that the mechanisms for induction of epidermal ODC activity by a complete carcinogen are quite different from those by which ODC activity is induced by tumor promoters. Furthermore, it has recently been shown that RA selectively inhibits TPA-induced synthesis of ODC protein and that it exerts its antineoplastic effect during tumor promotion by TPA by affecting the differentiation rather than the mere proliferation of mouse epidermal cells.[110,111] This last result is in keeping with the finding that retinoids induce transglutaminase (TG) activity in cultured mouse epidermal cells.[112,113] It should be recalled here that TG enzyme catalyzes the formation of ϵ(γ-glutamyl)-lysine crosslinks in specific membrane-associated proteins; these crosslinks result in a rigid and highly insoluble epidermal membrane that is associated with the differentiated state of the cell, whereas basal cells have very low TG activity. However, one can not extrapolate from this that retinoids always prevent cancer, because treatment with RA was ineffective in reducing ODC activity in some virally transformed cultured cell lines.[114]

Two new, physiological inhibitors of TPA-induced ODC activity in mouse epidermis in vivo have been identified and they are epidermal chalone and 1α,25-dihydroxyvitamin D_3.[115,116] We are at loss to explain the mechanisms of the inhibition of epidermal TPA-induced ODC activity by these two substances. However, in our laboratory we also found that the rabbit hepatic chalone selectively inhibited hepatic ODC enhancement during rat liver regeneration.[117] 1α,25-Dihydroxyvitamin D_3 strongly inhibited tumor formation in the two-stage carcinogenesis protocol in mouse skin.[118]

In conclusion, (1) the wealth of research with the two-stage carcinogenesis protocol of mouse skin, both in vivo and in vitro, can be divided into two different subsets. The first was devoted to clarifying the molecular mechanism(s) involved in the enhancement of polyamine biosynthesis observed in this type of experimental carcinogenesis, while the second, which is at present much smaller, was devoted to investigating the abnormalities of polyamine biosynthesis regulation in this type of experimental carcinogenesis. We think it would be naive to look for a role of polyamines specifically and exclusively in neoplastic growth, because they are growth-promoting substances in the broadest sense of the word and therefore they are very useful for studying any type of growth, both curbed and uncurbed. Therefore, if neoplastic growth has a specific effect on polyamine biosynthesis, it is certainly the deregulation of this metabolic pathway and such a deregulation is the biochemical precondition for the quantitative increases, when present, of polyamines in skin two-stage carcinogenesis. (2) The ODC induction in the epidermal cells by complete carcinogens seems to be caused by mechanism(s) different from those underlying ODC induction by tumor promoters. (3) ODC appears to be closely involved in both epidermal cell differentiation by TPA and in the hyperplaseogenic response to MZ. Epidermal hyperplasia is important for the completion of the promotion stage.[119] (4) Although the already-known concept that some compounds that induce epidermal ODC activity are weak tumor promoters or not tumor promoters at all (for review, see Reference 14), ODC seems to be so strictly related to tumor promotion that this biochemical parameter has been widely employed for screening new compounds as possible new tumor promoters.[120-124] We would, therefore, define this type of marker as sensitive for rather than as specific for tumor promotion.

However, two more important speculations can be made. (1) It is well known that the two-stage carcinogenesis protocol causes primarily clonal benign papillomas, with little probability that the papillomas will grow autonomously and develop into carcinomas.[125] Malignant conversion from skin papillomas into skin carcinomas is increased only by initiators, i.e., the complete carcinogens, but not by tumor promoters.[126] All these facts are cautions against drawing general and definite conclusions about the biological significance of polyamines in this type of experimental carcinogenesis. (2) Finally, the interpretation of the two-stage epidermal tumorigenesis was recently discussed differently by Hennings and Yuspa,[127]who suggested that stage I of promotion may occur spontaneously with time after initiation. In line with this, MZ can be an effective promoter when applied after DMBA at a much longer interval than that used for TPA.[127] Therefore, promotion stage does not seem to consist of two mechanistically distinct stages. Furthermore, the interpretation of two-stage carcinogenesis protocol in skin is even more complicated by the report that expression of genes related to the Harvey Murine Sarcoma virus (ras^H), capable of transforming NIH 3T3 cells, can be recognized in both carcinomas and papillomas obtained with this model of experimental carcinogenesis.[128,129] An alteration of the ras^H gene is therefore enough to initiate mouse epidermal carcinogenesis, which supports the view that in this model of experimental carcinogenesis chemical carcinogenesis and protooncogene expression are somehow connected and integrated. Possible synergistic interaction between tumor promoters and activated cellular oncogenes during multistage chemical carcinogenesis is further supported by the finding that TPA and teleocidin, but not phorbol, markedly enhanced the transformation of C_3H 10 $T^1/_2$ mouse fibroblasts when these cells were transfected with the cloned human bladder cancer c-ras^H oncogene.[130]

B. Carcinogenesis in Rodent Liver

For nearly 50 years, rodent liver has increasingly been used to study chemical carcinogenesis and, due to its versatility, this organ has become one of the most widely investigated in vivo models, and a variety of protocols for inducing liver cancer has been introduced.[12,22,23] Most of our current knowledge about the biochemistry of tumors rests on results from this kind of experimental carcinogenesis, despite some of its pitfalls. In vitro systems for carcinogenesis of hepatocytes have not provided so far the information needed for detailed analysis of the sequential biochemical events implicated in cancer development in vivo. Therefore, the in vivo rodent liver model is at present in no danger of being discarded. Generally speaking, rodent liver has many advantages, among which the most important are the following: (1) this organ consists of a population of cells in essentially G_0 that can be, at will, easily turned on to proliferate by a variety of different growth stimuli and (2) putative prestages of cancer, namely presumed targets for tumor promotion have been identified and are usually found long before overt cancer appears.[19,24] In other words, in the rodent liver system discrete focal new cell populations emerge sequentially and, accordingly, nodules (with a common metabolic pattern) formed during hepatocarcinogenesis process are focal collections of growing initiated cells.[12,17,22]

The multistep nature of cancer development is actually quite evident in the rodent liver model too.[24] Several agents promoting development of liver tumors have been identified.[131] Generally speaking, the two-stage process of epidermal carcinogenesis and that of hepatocarcinogenesis are very similar.[24] The largest difference is the easy and very early demonstration during hepatocarcinogenesis of the initiated cells (neoplastic nodules), long before the actual appearance of hepatic neoplasia, whereas no comparable morphologically identifiable structure has been described in epidermal carcinogenesis.[24] However, the ‘‘dark cells,’’ the targets for tumor promoters (see Section A above) in epidermal carcinogenesis, might somehow fill this gap between these two models of two-stage carcinogenesis.

Since much material about polyamine biosynthesis and content in chemically induced hepatocarcinogenesis or in fully developed hepatomas has already been reviewed (see Reference 14), during recent years attention has been given to the effects on polyamine biosynthesis and content of a variety of liver tumor promoters, some of which exhibit relatively high organ specificity, some not.[131] In any case, the hepatocyte proliferation is stimulated by virtually all compounds that promote tumor development in the liver.[131]

Application of TPA to skin has been shown to induce hepatic ODC activity in the mouse with the same time course as in mouse epidermis.[132] The same phenomenon, i.e., induction of hepatic ODC activity, was observed in both mice and in rats, after i.p. injection with TPA.[133-137] As in mouse skin, hepatic ODC induction by TPA is abolished by pretreatment with cycloheximide or actinomycin D, indicating the requirement for *de novo* protein and RNA synthesis.[134,135,137] However, treatment with TPA did not stimulate DNA synthesis in rat liver.[134] TPA-induced ODC activity was also observed in livers of hypophysectomized rats.[133] Increases in rat hepatic levels of the three main polyamines, putrescine, SPD, and SP, have been observed after TPA, i.p.[138] Although it may be assumed that for ODC induction by TPA in rodent livers gene activation must occur, the potential of TPA as a true tumor promoter in the mammalian liver has not been determined at this time.

The in vivo effects of other well known liver tumor promoters, i.e., phenobarbital (PB) and anthralin, on polyamine biosynthesis and/or content in rat liver are very similar to the effects of TPA, that is, ODC activity is induced and this induction is accompanied by accumulation of putrescine, SPD, and SP.[135,138-140] The time course of ODC induction by anthralin is slower than that after TPA.[139] Interestingly enough, barbital and PB, which have been shown to be more potent inducers of hepatic ODC activity in the rat than other barbiturates, were also the only barbiturates able to enhance the appearance of γ-glutamyl-transpeptidase (γ-GT)-positive foci in the liver of diethylnitrosamine (DENA)-treated rats.[140] This strict correspondence between the ability of some barbiturates to enhance the incidence of γ-GT-positive foci after DENA initiation and their ability to induce hepatic ODC activity in the rat further supports the idea that the ODC induction might be a suitable molecular marker for the hyperplastic response of rodent liver to tumor promoters. PB induced hepatic ODC activity even when administered in the diet, provided that the rats had previously been given DENA, i.p.[141]

When administered i.p., chloroform induced the activity of both species of ODC in the cytoplasm of the liver of the rat strain used.[142,143] This raises once again the question as to whether there might be multiple forms of ODC in the nonproliferating rodent liver too or whether the multiple forms are only present in proliferating rodent liver (for review, see Reference 14). Pereira et al.[142] showed that the quantitatively most important form of hepatic ODC has a short half-life, whereas the quantitatively less important form has a much longer half-life.

3-Methylcholanthrene (MC), injected i.p., induced both ODC activity (within a short time after the injection) and AHH activity (after a much longer time) in mouse liver.[136] A tumor promoter only, such as TPA, effectively induced hepatic ODC activity in mouse, but was totally ineffective in raising AHH activity.[136] The simultaneous injection of MC and TPA resulted in a synergistic induction of hepatic ODC activity and in a surprising inhibition of hepatic AHH induction.[136] On the ground of these results, these investigators concluded that the regulatory factors involved in hepatic ODC induction by TPA and carcinogenic polycyclic aromatic compounds are different.[136]

Single i.p. injections of 2-acetylaminofluorene (AAF) or of its hydroxy derivatives induced hepatic ODC activity in rats.[144] Furthermore, during chronic feeding of AAF to rats to obtain hepatoma, hepatic putrescine and SPD levels were increased, but not SP, which was, instead, markedly decreased.[145] In rats chronically fed AAF, the rise in hepatic γ-GT activity and the appearance of morphologically recognizable nodules in the liver was preceded by a rise in hepatic ODC activity.[146] Alterations in the activities of these two marker enzymes are temporally correlated with the time of nodule appearance and therefore are valuable reference and comparison points for studies of the biochemical mechanisms of hepatocarcinoma development. Moreover, ODC from both preneoplastic nodules and hepatomas from AAF-fed rats showed great stability, with little loss of activity during long-time incubation with normal rat-liver microsomes.[147] On the contrary, hydrocortisone-induced hepatic ODC from normal rats rapidly lost activity during long-time incubation with corresponding microsomes.[147] However, other investigators cast some doubts on this result, since they found no difference between ODC from normal liver and ODC from Morris hepatoma in the loss of enzyme activity when the partially purified ODCs were incubated with liver microsomes.[148] Nevertheless, in our opinion no comparison can be made between the two experimental hepatomas used by two teams, because the hepatoma used by Gravela et al.[147] is AAF-induced and therefore highly malignant, whereas the hepatoma used by Seely et al.[148] is Morris 7777, which is a well-differentiated hepatoma of low-grade malignancy.[149]

Olson and Russell[150] investigated the hepatic ODC levels in rats in which hepatoma development was induced by the model of Solt and Farber.[151] Despite some fluctuations hepatic ODC levels of treated rats were always significantly high, not only after the first DENA administration but also during the subsequent period of AAF feeding, and especially after partial hepatectomy.[150] Surprisingly, these authors found that AAF feeding per se did not at all modify hepatic ODC levels.[150] This discrepancy from the results of the other groups might be due to the low AAF concentration in the carcinogenic diet used by Olson and Russell.[150] Olson and Russell[150] considered prolonged induction of hepatic ODC activity to be necessary for the development of preneoplastic foci and the eventual formation of hepatomas. It is worth noting that hepatic γ-GT activity began to rise when the preneoplastic nodules began to appear, i.e., 25 days after the initial DENA administration and 4 days after partial hepatectomy, in rats given complete carcinogens-partial hepatectomy by sequential treatment.[150] Thereafter, the high hepatic γ-GT activity level was maintained for the duration of the experiment.[150] Therefore, we can conclude that there is quite good parallelism between hepatic ODC activity levels and hepatic γ-GT activity levels in this model of hepatocarcinogenesis too, except for the first time period after DENA administration, in which hepatic ODC activity rose and hepatic γ-GT activity did not.

A single injection of DENA into newborn female rats caused permanently high hepatic levels of SPD and SP, but the hepatic putrescine level was elevated only much later.[152] The polyamine levels were also high in the kidneys of these DENA-treated rats, but, surprisingly, they were higher in the left kidney than in the right, confirming the histology, which revealed only slight damage in the right kidney.[152] Substantially similar results were obtained by the same group when they investigated hepatic and renal ODC levels in newborn female rats injected once with DENA.[153] DENA was shown to induce ODC activity in a primary monolayer culture of adult rat liver cells also, while structurally similar noncarcinogenic compounds did not induce ODC activity at all.[154]

Recent studies have demonstrated that the sustained proliferation of peroxisomes and the subsequent hepatomegaly induced by some drugs, called peroxisome proliferators, lead to the development of hepatocellular carcinomas.[155] Therefore, some hypolipidemic drugs with hepatic peroxisome proliferative activity have been tested for their capacity to induce hepatic ODC activity in rats.[156] Wy, [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid was shown to be the most effective, after both a single injection and when administered chronically by feeding.[156] However, an initiating treatment with DENA followed by Wy feeding did not additionally increase the hepatic ODC levels over the levels observed in livers of rats fed Wy only, at least for up to 8 weeks after the beginning of the combined treatment.[156] Because of this and because hepatic ODC levels did not further increase in rats fed Wy for up to 15 weeks after DENA initiation, i.e., until that time when preneoplastic nodules and hyperplastic foci were observed, the authors concluded that hepatic ODC induction is associated with Wy-induced hepatomegaly rather than with the different steps of hepatocarcinogenesis.[156]

Further information has been obtained about the mechanism(s) involved in ODC regulation in livers of rats treated either acutely or chronically with hepatocarcinogen(s). A single injection of thioacetamide (at a noncarcinogenic dose) caused a severalfold increase in both immunotitratable ODC protein and polysomal ODC-mRNA activity.[157,158] Whereas there was coincidence of the peak for ODC protein increase and the peak for ODC activity in livers of thioacetamide-treated rats, the amount of hepatic enzyme protein increased during the treatment much less than the hepatic enzyme activity.[157,158] However, the peak for the hepatic ODC-mRNA activity was reached several hours before the peak for the hepatic ODC activity in thioacetamide-treated rats.[157,158] Moreover, in contrast to albumin-mRNA activity, hepatic ODC-mRNA activity was shown to be associated mostly with the free polysome fraction and this is in agreement with the cytosol location of ODC.[157,158]

All these results clearly indicate an involvement of translational or posttranslational modification of enzyme synthesis in the induction of hepatic ODC activity by thioacetamide.[158] Thioacetamide, when administered at a noncarcinogenic and nonnecrotizing dose, causes an additional derangement of ODC regulation in rat liver. This derangement is seen as a transient decrease in the activity of hepatic ODC antizyme (ODC-AZ), whose formation was elicited by i.p. putrescine injection.[159] This decrease is temporally concomitant with the well-known induction of hepatic ODC activity by this drug.[159] However, the time required for the restoration of normal ODC-AZ activity in livers of thioacetamide-treated rats is much longer than that required for restoration of normal hepatic level of ODC activity.[159] In another study, complementary to the preceding one in some respects, hepatic ODC-AZ activity in rats continuously fed 3′-methyl-4-dimethylaminoazobenzene (MDAB) was determined at regular time intervals until full development of hepatoma.[160] At all times the hepatic ODC-AZ activity in MDAB-fed rats was always significantly less than in normal resting rat liver and this reduction was not due to slower synthesis of ODC-AZ in hepatocarcinogen-feeding rats.[160]

All these results strongly suggest that the increase in hepatic ODC activity in thioacetamide-treated or MDAB-fed rats is also the result of defective control by the brake mechanism(s) (in this case ODC-AZ) of hepatic ODC activity and the high levels of putrescine constantly observed in the livers of thioacetamide-treated or MDAB-fed rats have little, if any, of their usual negative control effects on hepatic ODC activity levels.[159,160]

One of the most important advances in recent years in the polyamine field is surely the identification and characterization of the polyamine interconversion pathway.[161,162] Polyamine interconversion is another route for putrescine formation in mammalian cells, by degradation of SPD through two enzymic steps, of which the first is acetylation of SPD and the second is oxidation of N^1-acetylspermidine. The first enzyme involved is called SPD N^1-acetyltransferase and the second enzyme is polyamine oxidase, which splits N^1-acetylspermidine to putrescine and *N*-acetyl-3-aminopropionaldehyde. In vivo administration of carbon tetrachloride produced a sharp increase in the concentrations of N^1-acetylspermidine and N^1-acetylspermine in livers of both rats and mice, whereas the concentrations of these N^1-acetylated polyamine derivatives in normal resting rodent livers and in rodent organs other than liver of carbon tetrachloride-treated rats were very low or even not detectable.[163-166] These results are mainly due to the increased activity of hepatic SPD acetylase in CCl_4-treated rats.[167] However, N^1-acetylspermidine does not accumulate to very high levels in the liver under the above experimental conditions, because it is readily converted to putrescine.[167] This leads to a further accumulation of putrescine in the livers of CCl_4-treated rats, because putrescine formed through polyamine interconversion is combined with putrescine formed through decarboxylation of ornithine, which route is also enhanced in livers of CCl_4-treated rats (for review, see Reference 14). Furthermore, the increase in hepatic SPD acetylase activity by CCl_4 is the result of synthesis of new enzyme rather than of the release of enzyme from a hypothetical inactive form, as seen in an increase in the amount of the enzyme protein.[167,168]

Increased activity of hepatic SPD N^1-acetyltransferase was also observed in rats injected with dialkylnitrosamines.[169] The consequences of this increase are marked modifications of hepatic-polyamine levels of treated rats: (1) putrescine content increased because of the conversion of N^1-acetylspermidine into putrescine; (2) SPD content increased, since N^1-acetyltransferase also uses SP as a substrate to form N^1-acetylspermine, which in turn is converted into SPD by polyamine oxidase; and (3) SP content therefore decreased.[169] The increases in hepatic SPD/SP acetyltransferase activity induced by dialkylnitrosamines and by CCl_4 are much more striking and happen earlier than those of hepatic ODC activity induced by the same drugs, especially at early times after the administration of hepatocarcinogens.[169,170]

To summarize, some additional details have been obtained in recent years about the derangements in rodent liver of polyamine biosynthesis and of the polyamine interconversion pathway during hepatocarcinogenesis or after a noncarcinogenic dose of a hepatocarcinogen. These details are the following: (1) there are multiple forms of ODC, although this is not a completely new finding (for review, see Reference 14); (2) there is good correspondence between ODC induction and γ-GT induction and the appearance of the preneoplastic nodules; (3) there is reduced ODC-AZ activity; and (4) there is increased SPD/SP N^1-acetyltransferase activity. It is reasonable to think that these derangements take place in the rodent liver as regular responses to chronic or acute administration of a hepatocarcinogen, because they have been observed after administration of a large number of hepatocarcinogens so far known, though obviously not all have been studied. It is worth noting that the increase in SPD N^1-acetyltransferase activity is also observed in cells or tissues stimulated to curbed growth, such as in PHA-stimulated lymphocytes and in regenerating rat liver.[161,164,171-174]

Furthermore, TPA has been shown to induce SPD/SP N^1-acetyltransferase synergistically when added with PHA to cultured lymphocytes and the behavior of this acetyltransferase strongly resembles that described in the previous section of this chapter for ODC, under the same experimental conditions.[172] The stimulation of SPD/SP N^1-acetyltransferase activity in eukaryotic cells also appears to be connected with cell growth, whatever the type of the growth, normal or neoplastic. It must be emphasized here that the two enzymes that provide eukaryotic cells with putrescine, namely ODC and SPD/SP N^1-acetyltransferase, change in a shared and coordinated way when the cells start to grow.

III. POLYAMINE METABOLISM IN CULTURED MAMMALIAN NEOPLASTIC CELL LINES

A. Different Hepatoma Lines

Studies of polyamine biosynthesis in neoplastic cultured mammalian cell lines have essentially dealt with finding a suitable composition of incubation medium for stimulation or inhibition of the polyamine biosynthetic pathway or with identification of new inducers in vitro of ODC activity.

TPA was shown to induce ODC activity in Reuber H-35 hepatoma cells (a line of minimally deviated hepatoma cells derived from an MC-induced rat hepatoma) and in a human hepatoma cell line.[100,175-177] Studies of TPA-stimulated ODC induction of cells at different stages of growth, i.e., comparing log-phase cultures to confluent or stationary-phase cultures, indicated increased responsiveness to TPA as the cultures approached 100% confluence.[175] In contrast, both cAMP and dexamethasone appeared to be most effective in inducing ODC activity in the nonconfluent rapidly growing cell cultures.[175,178] The same type of growth-phase-dependent ODC induction has been reported for different cultured hepatoma cell lines when ODC was induced by the addition of ice-cold growth medium.[179] When a series of various phorbol ester derivatives were tested for their abilities to induce ODC activity in H-35 cells, their potencies for promoting tumor formation in mouse skin paralleled well their abilities to induce ODC activity, which means that nonpromoting agents were not able to stimulate ODC activity at all.[176]

Quite surprisingly, the less dense cultures synthesized more putrescine in response to TPA, with less assayable ODC activity, than the confluent cultures.[177] This is reflected by an inverse correlation between ODC activity and intracellular putrescine levels in response to TPA, the former increasing as the latter decreases during progression from rapidly growing or log-phase cultures to stationary-phase or confluent cultures.[175,177] However, when all of the cultures were supplied with suitable exogenous ornithine, they responded identically in terms of putrescine biosynthesis and ODC activity to TPA stimulation.[177] Therefore, Reuber H-35 hepatoma cells have apparently limited supplies of the substrate ornithine for putrescine synthesis.

EGF, like TPA, was also able to induce ODC activity in a human hepatoma cell line.[100] More importantly, when TPA and EGF were incubated simultaneously with the human hepatoma cultures, their effects on ODC activity were additive.[100] All these results further emphasize the important role, not yet completely clarified, of EGF in neoplastic transformation and growth.[180,181]

Arginine by itself induced ODC activity in H-35 rat hepatoma cells.[178,182] However, the composition of the culture medium is critical for the ODC stimulation in H-35 hepatoma cells elicited by different substances.[178] ODC induction in cells maintained in the salt/glucose solution differs from that of cells maintained in Eagle's minimal essential medium, since (1) neither cAMP nor dexamethasone stimulated ODC activity in cells maintained in the salt/glucose solution and (2) the kinetics of the asparagine-induced increase in ODC activity in cells maintained in salt/glucose solution profoundly differ from those in cells maintained in Eagle's minimal essential medium.[178] Furthermore, the well-known ability of asparagine to enhance ODC in mammalian cultured cells (for review, see Reference 14) is also shown by the finding that induction of ODC by insulin in different hepatoma cell lines is enhanced by the presence of asparagine in the culture medium.[182,183] Calcium in the culture medium is also important for inducing ODC activity in cultured hepatoma cells, because addition of a calcium-chelating agent, EGTA, inhibits ODC induction by cAMP.[184]

All these results revolve around ''conditions'': (1) what conditions are optimum for the induction of a single parameter, e.g., ODC, in a given cell line, but not for the induction of the same parameter in another cell line and (2) what conditions are optimum for a given parameter in a given cell line but might be either optimum or unfavorable for another parameter, such as DNA synthesis or induction of another enzyme, e.g., tyrosine aminotransferase.

There are conflicting reports on the presence of multiple forms of ODC in experimental hepatoma cells. From a cultured hepatoma cell line Mitchell and co-workers[185,186] separated two distinct forms of ODC, of which form I may be derived from form II by a specific proteolytic removal of a small peptide. Polyamines, but not putrescine, were found to stimulate the conversion of the more stable form II into the less stable form I.[185,186] Other investigators[148] were able to find only one form of ODC in Morris hepatoma 7777, but, as noted in the previous section of this chapter, this type of hepatoma has a low degree of malignancy and therefore it is not valid to compare the two situations.[149]

The acetylated polyamines, N^1-acetylputrescine, N^1-acetylspermidine, and N^8-acetylspermidine were found to induce ODC activity in cultured hepatoma cells.[187,188] However, other investigators reported that N^8-monoacetylspermidine, which, unlike N^1-acetylspermidine, is not a substrate for polyamine oxidase and undergoes enzymatic deacetylation, decreased ODC activity in cultured hepatoma cells, in spite of the fact that N^8-monoacetylspermidine is taken up by hepatoma cells much less than N^1-monoacetylspermidine.[189]

New insights into the role of ODC-AZ in ODC regulation were gained through studies with cultured neoplastic cells. The amounts of ODC-AZ complex were very small in confluent monolayers of hepatoma cells after the addition of fresh growth medium (which procedure is well known to strongly induce ODC activity in cultured cells).[190] Conversely, the amount of ODC-AZ complex in cultured hepatoma cells began to increase as ODC activity began to fall.[190] Similarly, a maximum of ODC-AZ activity (revealed by treatment with putrescine) was observed in cultured Ehrlich ascites tumor cells (a tumor not derived from liver cells) during mid-S phase, when the ODC activity had reached its minimum.[191,192] Furthermore, the half-life of ODC activity decreased as the ratio of the ODC-AZ complex to total ODC activity increased in hepatoma cells after the change of medium.[193] It is therefore quite clear that the regulation of ODC inactivation by ODC-AZ is a major factor in eliciting both the rapid increase and the subsequent rapid decrease of ODC activity in cultured hepatoma cells in response to inducing stimuli.

The possible involvement of polyamines in the initiation of DNA synthesis of cultured rat hepatoma cells has been reviewed.[194]

B. Different Nerve Tumor Cell Lines

The influence of composiiton of the culture medium on ODC induction and/or ODC regulation in cultured cell lines derived from tumors of nervous tissues, mainly neuroblastoma, glioma, and pheocromocytoma, has also been investigated. ODC induction in neuroblastoma cells incubated in salts-glucose medium has been studied to determine changes in ODC activity independent of the growth-associated substances usually contained in routine cell culture media.[195] ODC activity can be enhanced in these cells by a variety of amino acid analogues including α-aminoisobutyric acid (AIB), that cannot participate in protein synthesis.[195] Therefore, there is no direct relationship between the enhancement of cellular protein synthesis and the ability of amino acids to induce ODC activity. The ability of AIB to induce ODC activity also provides us with a probe for the involvement of A-system of amino acid transport in the regulation of ODC activity in neuroblastoma cells.[195] Na^+ is also required for the stimulation of ODC activity.[195]

In another paper from Canellakis's group, the various amino acids are classified in terms of their ability to induce ODC activity and of their effects on ODC activity, which had been induced previously with L-asparagine.[196] Group A amino acids (e.g., glutamic and aspartic acids) enhance the ODC activity level induced by asparagine, but do not elicit ODC activity by themselves.[196] Group B amino acids (e.g., L-phenylalanine and L-glycine) have little, if any, effect on ODC activity by themselves and do not affect the ODC activity elicited by asparagine.[196] Group C amino acids (e.g., D,L-alanine and glutamine) have intermediate effects; group D amino acids (e.g., L-proline, L-valine, L-isoleucine, L-arginine, and L-methionine) strongly inhibit ODC activity elicited by asparagine and have by themselves no effect on ODC activity levels, with the only notable exception being AIB.[196] Furthermore, analysis of ODC activity as a function of intracellular asparagine or AIB concentration shows that there are definite threshold levels of inducing amino acids for ODC induction.[196]

In the same neuroblastoma cell line, the specific transport system for all three naturally occurring polyamines was characterized.[197] This transport system shows the following features: (1) it is distinct from any other known nutrient transport system; (2) it is an Na^+-dependent process; (3) it is sensitive to sulfhydryl reagents; (4) it is sensitive to intracellular polyamine content; and (5) it is stimulated by amino acids that use the Na^+-dependent transport system, particularly asparagine.[197] It is worth noting that the electrochemical gradient in animal cells and tissues has been shown to be a major source of energy for the active transport of neutral amino acids (system A and ASC) and D-glucose. Furthermore, the possibility that amino acids transported by A-system, particularly asparagine, are coupled to intracellular polyamine level regulation via regulation of ODC activity and polyamine transport appears of paramount significance for better identification of the role of polyamines in cell growth.

As has been described for hepatoma cultured cells, the presence of calcium in the medium was also critical for ODC induction in rat glioma cells by calf serum.[198] However, the calcium dependence of the ODC induction is not associated with RNA synthesis.[198] EGTA, a well-known calcium chelator, inhibited both the basal and polyamine-stimulated ODC-AZ activity in rat glioma cells.[199] Furthermore, in cultured neuroblastoma x glioma hybrid cells, opiates decreased both cellular cAMP and ODC levels, suggesting that in this cell line too the ODC regulation is cAMP-mediated.[200] In 9L rat brain tumor cells, polyamine levels were lowest early in the G_1 phase and increased progressively as cells progressed from G_1 to G_2/M.[201]

TPA, EGF, and NGF caused common and similar biological responses in cultured rat pheochromocytoma cells (PC-12) and this polymorphic response included the ODC induction.[183,196,202-207] Parenthetically, TPA has been shown to induce ODC activity in mouse brain in vivo, but only during the early neonatal period.[208] The phenomenon of ODC induction in PC-12 cells has been characterized in more detail. First, NGF causes cell proliferation to decrease, followed by marked hypertrophy, whereas EGF enhances cell proliferation and does not induce cell hypertrophy.[203] Second, the effect of TPA on ODC activity was additive or more than additive to the effects of NGF and EGF.[204] Third, pretreatment with NGF reduced both EGF binding and ODC induction by EGF without modifying the effectiveness of TPA for inducing ODC activity.[203,204] Fourth, when PC-12 cells were incubated in salt-glucose solution, neither NGF nor EGF induced ODC activity, but these two growth factors were able to elicit an ODC response when an inducing amino acid, e.g., asparagine or AIB, was present in the culture medium.[183,196] Fifth, at least two distinct mechanisms seem to mediate ODC induction by NGF, one of which is cycloheximide insensitive and the other is dependent on protein synthesis.[207] Amazingly enough, NGF and EGF have moderate inductive or even sometimes inhibitory effects on *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50)(AMD) activity.[206] Furthermore, the changes in the levels of both the polyamine biosynthetic decarboxylases in PC-12 cells brought about by NGF and EGF are not matched by expected changes in the intracellular levels of the polyamines themselves.[206] Additionally, many benzodiazepines increase ODC activity level in PC-12 cells in the absence of NGF.[209]

C. Mouse Lymphoma and Leukemic Cell Lines

Glucocorticoids, such as dexamethasone and hydrocortisone, inhibit ODC activity in mouse S49 lymphoma.[210,211] This means that this neoplastic cell line still is sensitive to the physiological negative control of growth of lymphoid cells by corticosteroids. Furthermore, steroid-mediated inhibition of ODC activity in S49 lymphoma cells appears to be specific and to require cytoplasmic glucocorticoid receptors and transfer to the nucleus of steroid-receptor complexes, because ODC activity in some S49 cell variants with defects in some steps of the glucocorticoid cellular pathway is not inhibited by dexamethasone.[210] A variety of agents, including β-adrenergic agonists, PGE_1 and cholera toxin, increasing intracellular cAMP content, and, of course, cAMP itself, have been shown to inhibit both ODC activity and AMD activity in S49 cells.[211,212] It is important to remember that both glucocorticoids and cAMP inhibit proliferation of S49 mouse lymphoma cells, with accumulation of the cells in G_1, suggesting a relationship between the decrease in ODC activity and the inhibition of cell growth.[211] A study of S49 lymphoma cells comparing time patterns of the negative effects of cAMP and dexamethasone on polyamine metabolism and content demonstrated that the inhibitory effect of Bt_2cAMP on polyamine biosynthetic decarboxylase activities occurs much more rapidly than that of dexamethasone.[211] The reverse is true for the decrease in intracellular polyamine content.[211]

The activities of both the polyamine biosynthetic decarboxylases were closely related to the proliferative status of cultured Nb2 node lymphoma cells, because stimulation of mitogenesis by prolactin induced both ODC and AMD activities and the pattern of ODC induction was biphasic, with the greater peak well before DNA synthesis in the G_1 phase, and the second peak coincident with the onset of DNA synthesis.[213]

Finally, additional evidence for the regulation of ODC activity by the plasma membrane was provided by L-1210 mouse leukemic cells, in which some ionophores and cationic local anesthetics prevented the enhancement of ODC activity induced by changing the medium.[214]

D. Ornithine Decarboxylase and Polyamine Levels in Cultured Mammalian Neoplastic Cells During Differentiation Induced by Different Chemicals

The investigations of the behavior of ODC activity level and polyamine levels in cultured mammalian neoplastic cell lines during differentiation induced by various chemicals represent one of the most meaningful advances in the polyamine field in these last 5 years. For the convenience of the reader, we have summarized in Table 1 all the information on this topic available at present, except for the data obtained with inhibitors of polyamine biosynthesis, because they will be discussed elsewhere in this book.

These data are puzzling, because there is no single trend of changes in ODC activity levels and in polyamine levels in neoplastic cells induced to differentiate. One reason for this might be the differences in the compositions of the culture media used, which we know

Table 1
ODC AND PA LEVELS IN CULTURED NEOPLASTIC MAMMALIAN CELLS DURING DIFFERENTIATION INDUCED BY DIFFERENT CHEMICALS

Differentiation inducer	Type of neoplastic cell	ODC or PA levels	Ref.
RA	Murine embryonal carcinoma F9 cells	↓ ODC	215
HMBA	Murine erythroleukemic cells (DS 19)	↑ ODC	216
Canavalmine	Murine erythroleukemia cells 745A	↓ Free SPD	217
TPA DMSO RA	Human HL-60 promyelocytic leukemia cells	↑ Putrescine;—ODC, AMD	218,219
TPA	Human HL-60 promyelocytic leukemia cells	↑ PA	220,221
TPA; RA	Human HL-60 promyelocytic leukemia cells	↑ ODC; ↑ putrescine	222
1α,25$(OH)_2D_3$	Human HL-60 promyelocytic leukemia cells	↑ ODC; ↑ putrescine; ↑ SAT	223
HMBA DMSO	Friend erythroleukemia cells	↑ ODC; ↓ PA; ↑ PA acetylation	224—226
HMBA	Friend erythroleukemia cells	↓ PA	227
TPA	Human neuroblastoma cells SH-SY5Y	— ODC	228
TPA + RA		↓ ODC inducibility	
Bt_2cAMP + IBMX	Mouse NB-15 neuroblastoma cells	↓ ODC; ↓ ODC inducibility; ↑ SP content; ↓ putrescine transport	229—231
Butyrate	F98 rat anaplastic glioma cells	↑ ODC	232
NGF		— ODC	
TPA; NGF	Rat pheochromocytoma PC12	↑ ODC	204
MSH	Cloudman S91 mouse melanoma cells	↑ ODC; ↑ TG	233, 234

Note: ↑ = increased; ↓ = decreased; — = no change; TG = transglutaminase; PA = polyamines; SP = spermine; SPD = spermidine; SAT = spermidine acetyltransferase; RA = retinoic acid; DMSO = dimethylsulfoxide; TPA = 12-*O*-tetradecanoylphorbol-13-acetate; IBMX = isobutyl-1-methyl-xanthine; and HMBA = hexamethylene bisacetamide.

to influence basal ODC levels and also the magnitude of ODC changes and consequently the subsequent polyamine levels in cultured mammalian neoplastic cells.

IV. POLYAMINES IN HUMAN ONCOLOGY

In recent years we have obtained no important new information about the clinical significance and usefulness of determinations of the polyamine levels in human oncology, i.e., either in human neoplasm samples or in physiological fluids of oncopathic subjects. Therefore, all the comments and the criticisms on this topic in the section of our previous review still substantially hold.[15] The reader is therefore referred to that review.[15] However, there are some new findings that merit some attention and discussion.

The polyamine contents of some groups of human neoplasms not investigated earlier have now been described. SPD and SP were strikingly high in primary colorectal cancers, but independent of the histological grade or Duke stage.[235,236] Furthermore, N^1-acetylspermidine levels were significantly higher in well- and moderately differentiated adenocarcinomas of human colon than those in adenomas and control mucosae.[236] Monoacetylated polyamines were also found to be significantly high in human breast cancers and virtually absent from normal breast tissue, although once again their levels were not correlated with the histological grade or the oestrogen-receptor status.[237] However, unacetylated polyamine levels in breast cancers correlate well with the indices of tumor aggressiveness, histological grade, and estrogen-receptor status.[238] Therefore, assay of polyamines in samples of human neoplasms seems to be, at the best, a biological indicator for tumor aggressiveness.

Elevated urinary levels of acetylated polyamines in patients with non-Hodgkin's lymphomas and melanoma have been reported.[239,240] The urinary levels of putrescine, SPD, and SP correlated well with the stage of the disease in patients with non-Hodgkin's lymphomas, with bladder cancer, with small-cell carcinoma of the lung, or with stomach cancer.[241-244] Surprisingly enough, the total amounts of urinary polyamines in patients with colorectal cancers were not significantly different from those of control subjects.[245] A preliminary but interesting study showed that the urinary excretion of 5′-methyl-thioadenosine (MTA), a by-product of polyamine biosynthesis, was not significantly increased in a group of patients with differing neoplasms, suggesting that MTA-phosphorylase (the enzyme-splitting MTA) is probably responsible for in vivo removal of this nucleoside inside the cells, thus overcoming the MTA overproduction that mandatorily accompanies the polyamine overproduction in the cancer patients.[246]

As for the polyamine levels in plasma of human beings with cancer, SPD concentrations were correlated with the response to the therapy in patients with myeloma but not in patients with malignant melanoma.[247,248]

Erythrocyte SPD and SP levels were significantly increased in patients with malignant hepatic tumors, with head and neck cancers or with bronchopulmonary cancers, especially in cases of squamous-cell carcinoma but not in cases of adenocarcinomas.[249-251]

Two studies in very recent times have confirmed our original finding that polyamine biosynthetic decarboxylase activites (especially ODC) in human neoplastic tissues are correlated quite well with the degree of malignancy and rate of growth of the tumor (for review, see Reference 15). Levels of ODC activity were measured in biopsy specimens of colonic mucosa and polyps from patients with familial polyposis.[252] Mucosal ODC values became increasingly higher going from morphologically normal, flat mucosa from patients with polyps to dysplastic polyps, with the values for adenomatous polyps intermediate.[252] This result supports the idea that ODC activity is a useful marker for familial colonic polyposis and for monitoring the progression to marked dysplasia. It also suggests that the higher the state of proliferation of the colonic mucosa the greater the potential for development of polyps and progression of adenomatous polyps to a dysplastic state. Similarly, ODC activity levels in human colon cancer were found to be higher than those of normal-appearing mucosa.[253] Those results, together with those previously reported by us (see References 27 and 28) indicate the considerable usefulness of assay of ODC in biopsy specimens from human neoplasms, especially for groups of tumors with well-defined grading and well-defined growth rate ranges.

For the connections between polyamines and human primary tumors of the central nervous system, the reader is referred to our very recent review on this topic.[254]

In conclusion, after considering all the papers published in the last 15 years about polyamine assays in physiological fluids in human oncology, we do not feel it is likely that further enlargment of the literature with more cases and more data and/or another analytical approach will make measuring the levels of polyamines (whether free or acetylated) in blood and/or urine of cancer patients appear more useful as indicators of tumor growth and, even less so, for detection of neoplastic disease. Instead, we think that measuring the levels of polyamine biosynthetic decarboxylases in biopsy specimens from human neoplasms can be very useful in clinical oncology for determining the degree of malignancy and, in any case, merit further study.

V. POLYAMINE METABOLISM IN CARCINOGENESIS INDUCED BY PHYSICAL AGENTS

A. Carcinogenesis Induced by UVB Irradiation

The UV spectrum, the part of the electromagnetic spectrum between visible light and X-rays, is divided conventionally into three major sections: (1) short-wave UV, with wavelenghts of 250 to 290 nm (also known as UVC or germicidal light), (2) midwave or sunburn UV, with wavelengths of 290 to 320 nm (also known as UVB), and (3) long-wave UV, with wavelengths of 320 to 400 nm (also known as UVA). It is widely accepted that the wavelengths most carcinogenic for the induction of experimental epidermal neoplasia are in the UVB range. UV irradiation from the sun is a major causative factor for human skin cancer and similar skin cancers can be induced in laboratory animals with a sun lamp or other UV source. Polyamine metabolism in mouse epidermis after UV irradiation has been recently reviewed.[255] Few other papers have been added in recent years.

Multiple irradiation with UVB results in a more rapid ODC induction and putrescine accumulation in the epidermis of hairless mice than does a single irradiation with UVB.[256] The reason for the earlier peaking of epidermal ODC activity after multiple UVB irradiations is unknown, but it is of interest in view of the substantial evidence in the literature for the hypothesis that the ODC induction and changes in polyamine levels in epidermis are an essential part of the early stages of skin carcinogenesis. No further increase in UVB-induced enhancement of epidermal ODC activity occurred when hairless mice were also given UVA irradiation, i.e., there is no photoaugmentation (photoaugmentation is the sensitization of the skin by long-wave UV irradiation to the effects of UVB).[257] This result is in keeping with the inability of UVA to induce epidermal ODC activity in the hairless mouse.[258] The results of Gange and Mendelson[257] are also consistent with the studies in mice showing that intermittent UVA irradiation is not carcinogenic and that UVA added to UVB in the ratio that is present in noon-time sunlight does not augment the carcinogeneic effect of UVB.

Topical application(s) of RA to hairless mouse skin significantly inhibited UVB induction of epidermal ODC activity when RA was administered either prior to UVB or repeatedly after UVB.[259,260] However, RA alone, applied topically to normal hairless mouse skin causes a dose-dependent increase in epidermal ODC activity, which is detectable only 24 hr or more after treatment.[261] Furthermore, RA treatments reduce the carcinogenicity of UVB to hairless mouse skin.[262] UVB-induced skin carcinogenesis in hairless mice was also inhibited by two different antiinflammatory agents, indomethacin and triamcinolone acetonide, applied topically.[263] All these results further support the hypothesis that ODC induction is an essential component in UVB skin carcinogenesis, as in chemically induced mouse skin carcinogenesis. However, treatment with RA only moderately inhibited ODC induction by germicidal UV light in primary mouse epidermal cell cultures, but it almost completely prevented the induction by TPA.[264] Therefore, RA is little, if at all, effective in inhibiting ODC induction in vitro, when this enzyme induction is caused by an agent, such as viruses or UV light, that acts in the cell independently from a specific receptor.[114,264]

B. Carcinogenesis Induced by Ionizing Radiation

There is only one report showing delayed ODC induction in mouse skin after a single irradiation with low-penetrating ionizing radiations, β rays.[265] Ionizing radiation should have an initiating action and is also suspected to have tumor-promoting activity. However, the kinetics of the induction of epidermal ODC activity by β rays in the mouse are markedly different from those of TPA.[265]

VI. EPILOGUE

In spite of the numerous results showing some important derangements of polyamine

Table 2
MAIN ABNORMALITIES OF POLYAMINE METABOLISM IN NEOPLASTIC CELLS

Types of abnormality	Ref
Multiple forms of ODC	185, 186, 266
Lengthening of the half-life of ODC	266—269
Increased ODC specific activity plus stabilization	101
Greater ODC stabilization to *in vitro* microsome-dependent inactivation	147
Greater increase of ODC activity in response to fresh media or addition of serum	270—274
Synergistic induction of ODC activity by TPA plus fresh medium	94, 95, 271, 275
Loss of ODC circadian rhythm	276
Reduced sensitivity of serum-dependent ODC induction to inhibition by exogenous putrescine	272—274, 277
Decreased activity of the ODC-antizyme in response to exogenous putrescine	159, 160
Reduced sensitivity of ODC to inhibition by retinoic acid	114
Defective ODC control by cytoskeleton	278
Increased activity of N^1-spermidine acetyltransferase	164, 165, 167—170
Increased putrescine transport	229, 277, 279, 280
Absence of or decrease in specific excretion of SPD	280—282
Changes in polyamine binding capacity of fibronectin	283

biosynthesis in some experimental tumors, in cultured neoplastic cells, and in some mammalian organs during experimental carcinogenesis, only very seldom are they mentioned in critical reviews of the biochemistry of cancer cells in general or of some experimental neoplasm in particular. We do not at all wish to eulogize in these concluding remarks the importance of polyamines in neoplastic growth. We wish only to bring to attention the nonancillary role of polyamines in the biochemical strategy used by neoplastic cells for uncontrolled growth.

A. The Programmed Deregulation of Polyamine Metabolism in Neoplastic Cells

It has become increasingly evident that modifications at three different levels or combinations thereof are needed for mammalian cells to become neoplastic. These are (1) modifications of gene structure; (2) modifications of external environment; and (3) modifications of the metabolic steps providing energy substrates and growth-promoting substances.[9,284] It is quite clear that the modifications of polyamine metabolism in neoplastic cells are of the third type. We can now understand how altered polyamine production in neoplastic cells provides a metabolic situation in which these cells have great amounts of the polyamines greatly required, together with other growth-promoting substances, for autonomous growth of tumors. From this point of view the qualitative derangements of polyamine metabolism in neoplastic cells are much more important than the quantitative ones, because the first make it possible to have an uninterrupted supply of polyamines for the cancer cells, leading eventually to their characteristic growth. It cannot, therefore, be considered fortuitous that most of the derangements of polyamine metabolism of cancer cells concern ODC, since the pivotal role of this enzyme in polyamine biosynthetic pathway is widely known (for review see Reference 14). Nevertheless, it would be restrictive to think that the derangements of malignant transformation and neoplastic status concerning polyamine metabolism are limited to ODC regulation. We have listed in Table 2 the abnormalities of polyamine metabolism regulation observed so far in cultured mammalian neoplastic cells and in mammalian tumors.

Although it is clear from Table 2 that the programmed deregulation of polyamine metabolism in cancer cells includes many aspects; many, if not most, of the tesserae of the mosaic are at present lacking and consequently the picture of the polyamine abnormalities in tumors is far from complete, as is our understanding of it. However, the abnormalities so far known are important enough, persistent enough that they must be of paramount importance in the biochemical strategy of the neoplastic cells, characterized, as mentioned in Section I, by widespread programmed deregulation involving many different metabolic pathways.

B. Autocrine Regulation of Proliferation of the Neoplastic Cell: A Role for Polyamines?

In addition to having escaped from the mechanisms normally controlling cell proliferation, transformed cells also require fewer exogenous growth factors for optimal growth and multiplication than do their normal counterparts.[180,285-291] It was suggested that this is because the autonomous growth of neoplastic cells (and indeed some other phenotypic characteristics of malignant transformation) is due to polypeptide growth factors which act on the same cells that produce them, after binding to appropriate receptors on the surface membrane. This process, in which secretory cells are activated by their own secretion products, has been called ''autocrine secretion'' and this may well become a central concept for understanding the biochemical bases of the aberrant growth of mammalian tumors.[288-297] Parenthetically, autocrine loop, in which a cell both secretes and responds to a soluble growth-promoting factor, is not limited to cancer cells, but it may also be operative during repair of injured tissue and during embryonic development.[290] The polypeptide growth factors released from many types of cultured tumor cells, so far identified, that function in this autocrine fashion and positively affect neoplastic cell growth include (1) Type α-transforming growth-factor (TGF-α), which shares amino acid sequence homology with EGF; (2) peptides related to PDGF; and (3) bombesin.[287,291-297] We should mention here that TGF-α and PDGF have been shown to induce ODC activity both in vivo and in vitro, and putrescine transport.[183,298,299]

Furthermore, the hormonal effects on the growth of endocrine-dependent mammary tumors grown in cultures include ODC stimulation and, even more important, polyamines closely interact with the autocrine control of mammary tumor growth by estradiol.[300-302] Therefore, it is obvious that the growth factors produced by neoplastic cells also utilize the polyamine biosynthetic pathway to induce cancer growth by augmenting the production and the availability of polyamines inside the cell. This role of polyamines as effector molecules between the autocrine growth factors and cell growth itself makes polyamines major factors, although by no means the only ones, for autonomy of growth.

Let us conclude this chapter with a provocative proposal: since it is widely known that the atherosclerosis is also characterized by an abnormal proliferation of arterial smooth muscle cells,[303,304] why not try to better understand the role of polyamines in neoplastic growth by increasing investigations of polyamine metabolism in development of atherosclerosis?[299]

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Chapter 13

POLYAMINES AS MARKERS OF MALIGNANCY

Takatoshi Matsumoto and Osamu Suzuki

TABLE OF CONTENTS

I. Introduction 220

II. Polyamine Assays in Clinical Medicine 220
  A. Cleanup Procedure 220
  B. High-Performance Liquid Chromatography and Amino Acid Analyzers 220
  C. Enzymatic Assays 222
    1. Total Assay 222
    2. Differential Assay 223
  D. Gas Chromatography 223
  E. Other Methods 224

III. Evaluation of Polyamines as Tumor Markers 224
  A. Characteristic Changes in Polyamines in a Specific Cancer 224
    1. Putrescine in Brain Tumors 224
    2. Putrescine and Spermidine in Hematological Malignancies 225
    3. Limitations in Solid Tumors 225
  B. Acetylpolyamines as Tumor Markers 225
  C. Materials Used for Analysis 226
  D. Other Factors Affecting Polyamine Levels 227
    1. Nontumor Diseases 227
    2. Physiological Factors 227
  E. Comparison with Other Tumor Markers 227

IV. Conclusions 229

References 230

I. INTRODUCTION

In 1971, Russell reported high urinary excretion of polyamines (including putrescine, cadaverine, spermidine, and spermine) in cancer patients.[1] This discovery made polyamines candidates for tumor markers. We think it timely to review methods of assays for polyamines and their usefulness and limitation as tumor markers in this chapter, because many data have now been accumulated to be evaluated.

II. POLYAMINE ASSAYS IN CLINICAL MEDICINE

A. Cleanup Procedure

Samples with high protein contents are usually deproteinized with trichloroacetic acid, perchloric acid, or sulfosalicylic acid;[2,3] then they are subjected to cleanup procedure prior to analysis with an equipment. The most classical method employed was the extraction with *n*-butanol.[4] The recovery of putrescine or spermidine is satisfactory with the organic solvent, but that of spermine and acetylpolyamines is relatively low. Various kinds of ion exchangers were also employed for separation of polyamines; Dowex-50,[5,6] Amberlite CG-50,[6-8] Bio-Rex 70,[9] P-cellulose,[10] Whatman P81 paper,[11] and CM-cellulose.[12,13] Recently, small silica gel cartridges (Sep-Pak or Chemco-Sep) have been found useful for preliminary isolation of polyamines and acetylpolyamines.[14,15] They seem very useful for their rapid isolation, but sometimes suffer from their low capacity. The C_{18} Sep-Pak cartridges were not useful for isolation of underivatized polyamines and acetylpolyamines (unpublished observation), but useful for that of their dansyl derivatives.[16]

B. High-Performance Liquid Chromatography and Amino Acid Analyzers

In 1976, Samejima et al.[13] reported a high-performance liquid chromatography (HPLC) method for polyamines. Since that time, a number of papers have appeared on their HPLC analysis. Methods reported from 1976 to 1980 are summarized in great detail in a review by Fujita et al.[17] We can count as many as 13 papers[14,16,18-28] on HPLC for polyamines published in recent 5 years (1981 to 1985) in our file. The methods are being improved to make them faster, more sensitive, and to get better resolution. Recent efforts have also been directed toward assays for acetylpolyamines.[14,18,26,27] Reversed-phase columns are now most popular.[14,16,19,21-28] The post-column eluates are generally derivatized with *o*-phthalaldehyde for fluorometric detection;[18,19,26-28] however, precolumn derivatization with dansyl chloride is also employed in many reports.[16,21,23,24] Two papers appeared on precolumn derivatization with *o*-phthalaldehyde.[22,25] The detection limit of the fluorometric HPLC is 1 to 10 pmol in an injected volume. Fluorescamine can also be used in place of *o*-phthalaldehyde, but the latter is now more popular because of its lower cost. In some recent papers, ion-paring agents such as octanesulfonic acid,[19,26,27] heptanesulfonic acid,[21] and triethylammonium phosphate,[23] were added to the mobile phase in the reversed-phase HPLC. This ion-pairing technique is a combination of advantages of reversed-phase and ion-exchange chromatographies, and thus is sometimes very useful to obtain much better resolution. The HPLC methods are used most widely for analyses of polyamines and acetylpolyamines mainly for pure research purposes.

In our laboratories, we are using a column (0.4 × 5 cm) of a CK-10U (Mitsubishi Kasei, Tokyo) cation exchanger, and obtaining good results for separation of 11 kinds of polyamines and acetylpolyamines as shown in Figure 1; a step-gradient system with 0.2 *M* citrate buffers is employed.

Many papers were published describing polyamine analysis with amino acid analyzers (AAA).[29-31] The latest is the report by Russell et al.[31] Polyamines can be detected either photometrically by ninhydrin reaction, or fluorometrically with *o*-phthalaldehyde. Even with

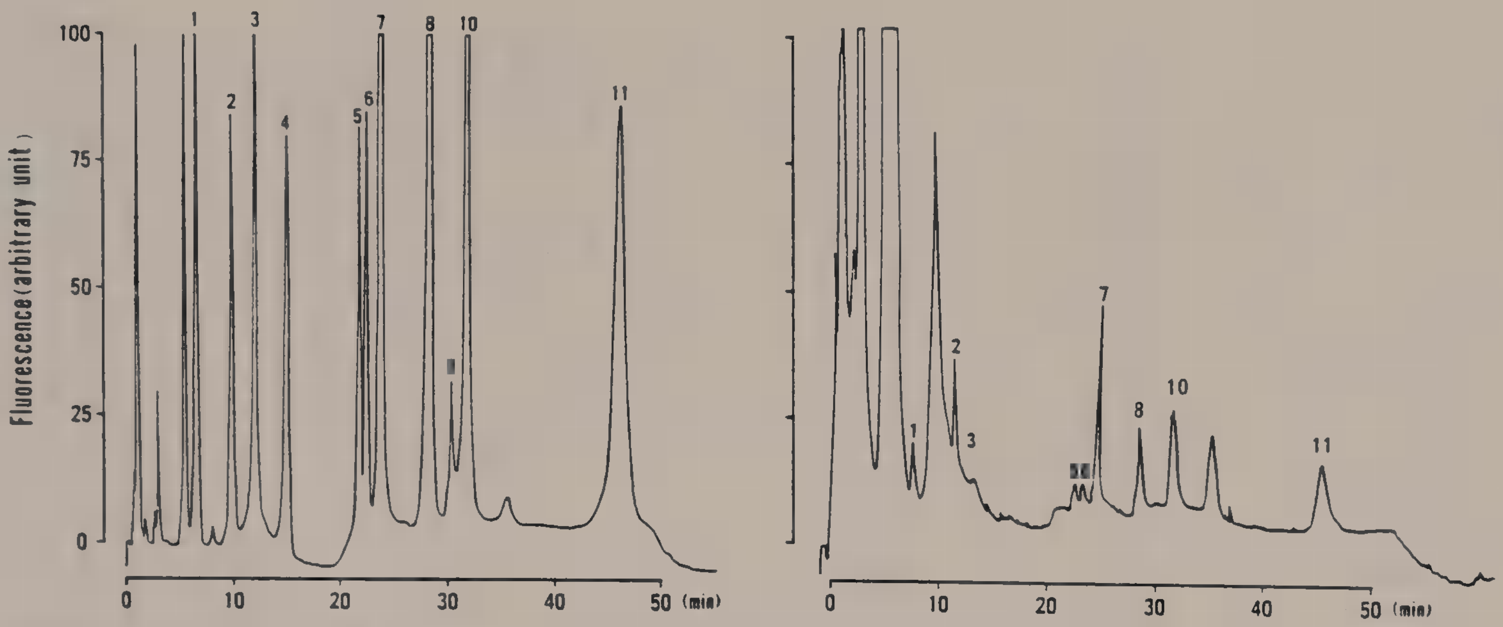

FIGURE 1. HPLC separation of the mixture of the authentic polyamines and their related compounds (left panel) and of an urine extract (right panel) of a cancer patient. The column used was CK-10U (0.4 × 5 cm). A three-step gradient system with 0.2 *M* citrate buffers containing 0.5, 1.0, and 2.5 *M* NaCl was used during 60 min. 1, acetylputrescine; 2, acetylcadaverine; 3, histamine; 4, 1-methylhistamine; 5, N^8-acetylspermidine; 6, N^1-acetylspermidine; 7, putrescine; 8, cadaverine; 9, N^1-acetylspermine, 10, spermidine; and 11, spermine.

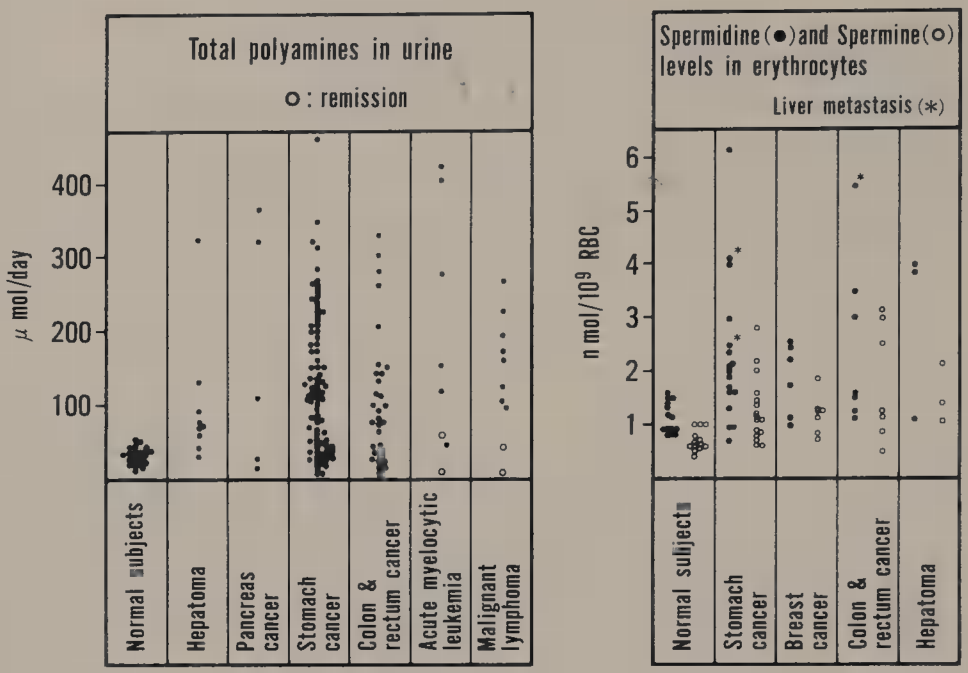

FIGURE 2. Total polyamines in urine, and spermidine and spermine in erythrocytes of normal subjects and cancer patients, measured by enzymatic methods.

the fluorometric detection, the sensitivity with AAA is about one order of magnitude lower than in HPLC, because of the longer columns used; the time required for measurements is several times longer than in HPLC. However, these disadvantages can be overcome to some extent by its high reproducibility and automatization. Nowadays, AAA methods are being replaced by HPLC methods.

C. Enzymatic Assays

In 1963, Bachrach and Oser[32] tried to develop an enzymatic assay for spermidine in solution or rat liver with a spermidine oxidase from *Serratia marcescens*. In the same year, Unemoto et al.[33] also reported an enzymatic assay for spermidine and spermine in rat liver after their separation with an ion exchanger resin using bovine plasma amine oxidase (EC 1.4.3.6); Bachrach and Reches[34] reported a similar but more sensitive method using the same enzyme. Harik et al.[35] reported an assay method of putrescine in tissues using activation of *S*-adenosylmethionine decarboxylase by putrescine. Braganca et al.[36] utilized a snake venom oxidase for the assay of polyamines in serum. However, none of the above papers dealt with clinical materials. Matsumoto et al.[7,8,15] first introduced enzyme assays into clinical studies, and then three papers by other groups appeared on similar subjects.[9,11,37]

1. Total Assay

In the body fluid samples from patients with solid cancers, more than two polyamines are usually elevated. The increase in a single specific amine is rare, probably because of interconversion among the amines in the human body.[38] Figure 2 (top panel) shows urinary excretion of total polyamines in normal subjects and cancer patients expressed as per day, measured by our enzymatic method.[8] In this assay system, soybean seedling amine oxidase active toward all polyamines was used. Our results showed that a simple enzymatic assay even for total amounts of the amines was useful for clinical screening and monitoring tumor activities. Kubota et al.[9] utilized an acetylputrescine deacetylase from *Streptomyces* in place of hydrolysis with HCl prior to an enzymatic oxidation of total polyamines, which appreciably shortened the assay time. Very recently, we also have partially purified an acetylputrescine deacetylase from *Micrococcus*,[39] which can be used for polyamine assays.[15] Fagerström et al.[40] and Bachrach and Plesser[11] used chemiluminescence for detection of hydrogen peroxide formed in the enzymatic reaction; it increased sensitivity 10- to 100-fold as compared with the photometric or fluorometric detection, although luminescence analyzers are required for the assays.

The enzymatic assays for total polyamines are simple and thus recommendable for a large number of samples. The cleanup with silica gel cartridges, followed by incubation with an amine oxidase in the presence of acetylputrescine deacetylase, seems the fastest procedure at the present time.[15] The photometric detection can be used for human urine, but fluorometric[7,41] or chemiluminescence assays[11,40] have to be used for serum and cerebrospinal fluid (CSF) because of the low polyamine contents in them. The photometric assays, of course, are most desirable for their widest distribution.

2. *Differential Assay*

If each polyamine can simply be measured by an enzymatic method, it seems very useful both for pure research and for clinical investigation. With use of the combination of different types of polyamine oxidases, differential assays for spermidine and spermine are possible. We employed oat seedling polyamine oxidase,[42] which attacks both spermidine and spermine, and barley seedling polyamine oxidase,[43] which attacks only spermine, in weak acid media. The spermidine levels can be calculated by subtracting the spermine value from the spermidine + spermine value. A detailed procedure for a differential enzymatic assay of spermidine and spermine in human tissues is described in our previous report,[15] and examples for erythrocyte spermidine and spermine are shown in Figure 2 (bottom panel). Total amounts of putrescine and cadaverine can easily be measured with diamine oxidases.[7] Differentiation between putrescine and cadaverine is theoretically possible; the oxidative product γ-aminobutylaldehyde formed from putrescine by the action of a diamine oxidase is easily cyclized to yield Δ^1-pyrroline which reacts with *o*-aminobenzaldehyde to produce a yellow color photometrically measurable.[32] Therefore, the value of cadaverine can be calculated by subtracting the putrescine value from the putrescine + cadaverine value. However, such a putrescine assay through Δ^1-pyrroline formation suffers from its low sensitivity because of the low molecular extinction coefficient of the color product (1.86×10^3 mol^{-1} cm^{-1}).[32]

Recently, Otsuji et al.[37] also reported a differential photometric assay for putrescine + cadaverine, spermidine, and spermine in human urine using polyamine oxidase from *Penicillium chrysogenum* and putrescine oxidase from *M. rubens*. This method cannot differentiate between putrescine and cadaverine. The enzymatic differential assays for polyamines await to be evaluated in the fields of both pure research and clinical investigation.

D. Gas Chromatography

Gas chromatography (GC) seems to be a useful tool for assays of polyamines and acetylpolyamines. Although GC seems a little more tedious than HPLC, it has a great advantage in that it can be connected to mass spectrometry (MS), which is most powerful to identify compounds. In addition, gas chromatographs are simple in their structure and principle, and thus enjoy high reproducibility and easy handling. Many papers were published on GC[44-50] and GC/MS[51-54] assays for polyamines and their derivatives in biological samples. GC/MS seems indispensable for discovery of a new polyamine derivative.

The sensitivity of GC can be increased with use of an electron capture detector,[44,47] nitrogen detector,[48-50] and mass fragmentography.[51,54] However, GC analysis of polyamines at low concentrations generally suffers from marked tendency of their adsorption to GC columns; peaks show remarkable tailing[44] and the calibration curves are not linear, especially in low concentration ranges. This problem ruins the highly sensitive detection systems. Spermine sometimes cannot be detected by GC due to its marked adsorption to columns even after efforts for its derivatization. Yamamoto et al.[50] had to use a special GC column (0.5% KT-

300) only for separation of spermine in addition to a general column for diamines and spermidine.

The solventless capillary GC[46,54] seems most promising for rapid and simultaneous analysis of polyamines and acetylpolyamines; it will minimize such adsorption and the time required for measurements. The capillary methods may be superior to the HPLC methods in sensitivity and resolution under their good conditions. The wide-bore capillary GC recently developed seems worthwhile trying for polyamines and their related compounds.

E. Other Methods

Radioimmunoassays and enzyme immunoassays are generally very sensitive and rapid, and thus very suitable for routine clinical investigation, if specific antibodies are available. In 1975, Bartos et al.[55,56] reported a radioimmunoassay for spermidine and spermine in human serum. However, each antibody cross-reacted by more than 20% between the polyamines. Later, they reported a fairly specific antibody against spermidine[57] or spermine.[58] In 1983, Fujiwara et al.[59] developed an enzyme immunoassay for spermidine and spermine; each antibody also cross-reacted between them to some extent. The most serious problem for the immunoassays is that no one succeeded in obtaining antibodies against putrescine. Clinical data are scant for immunoassays.

The use of thin-layer chromatography (TLC) for quantitation of polyamines and acetylpolyamines seems now somewhat outdated;[20-24,60] it should be used for preliminary identification and semiquantitation of the amines.

III. EVALUATION OF POLYAMINES AS TUMOR MARKERS

After the discovery of increased polyamine excretion in cancer patients by Russell,[1] many papers were published on clinical studies on polyamines. The most enthusiastic studies seem to have been done around 1975. However, during the time when limitations of polyamines as tumor markers became more and more evident, the interest in this subject declined especially in the U.S. until 1980, because of the difficulty of obtaining grant supports for such studies. However, it seems too early to have lost its interest because of insufficient data accumulated until that time. Some excellent reviews on the utility of polyamines in clinical investigations appeared about 5 years ago.[61-65] During the past 5 years, a considerable number of papers have been published on the clinical aspects of polyamines. One of the best examples is the proceedings of an international symposium held in Gifu, Japan in 1984;[66] nearly half the number of papers presented at the symposium were devoted to clinical studies. We, therefore, try to mainly cover new literature published in the past 5 years in this section.

A. Characteristic Changes in Polyamines in a Specific Cancer

Putrescine, spermidine, and spermine, of course, tend to increase in body fluids of cancer patients, and their increase becomes more marked in more malignant, active, metastatic, and advanced cancers. However, many reports showed that polyamine measurements are disappointing for early diagnosis of cancers. In recent reports on clinical studies of polyamines, therefore, attention has been directed toward longitudinal studies, to follow up efficacy of therapies, to predict response to therapies, and to detect relapse of cancers.

There is poor regularity as to which polyamine is elevated in a specific cancer, probably because of easy interconversion among them.[38] In spite of the great diversity of the data accumulated up to now, some trends of changes in polyamines for specific tumors should be pointed out.

1. Putrescine in Brain Tumors

The CSF polyamines in brain cancers were well studied by Marton et al.[67,68] They reported that CSF putrescine showed very good correlation especially with medulloblastoma and glioblastoma, and was useful for predicting recurrence before any other available diagnostic technique; there were no false positives. Miyazaki et al.[69] have recently supported the idea of Marton et al.[67,68] with experimental brain tumors in rats. The clinical utility of CSF putrescine in brain tumors should preferably be confirmed by other groups, because such studies have been conducted almost exclusively by Marton's group. In contrast, CSF spermidine was reported to be a much better parameter than putrescine in central nervous system leukemia.[70] Recently, Volkow et al.[71] have suggested that ^{11}C-putrescine can be used for imaging of brain tumors by positron-emission tomography, based on the preferential uptake of putrescine by animal brain tumors.

2. *Putrescine and Spermidine in Hematological Malignancies*

It is well known that there is a good correlation between polyamines and hematological malignancies including leukemias, malignant lymphomas, and multiple myeloma.[63] All polyamines were significantly elevated in urine of leukemia patients with factors (fold) of 1.2 to 4.1 for putrescine, 2.4 to 9.6 for spermidine, 1.9 to 29.3 for spermine, and 2.0 to 5.2 for acetylspermidine.[63] Many years ago, Russell[72] proposed an idea, based mainly on studies on hematological malignancies, that a sharp rise of spermidine in serum or urine observable within 72 hr after the initiation of chemotherapy can predict the response to therapy; the higher rise of spermidine is followed by better therapy. She also suggested that spermidine reflects tumor cell kill (necrosis) and putrescine the tumor growth;[62] thus putrescine should be the first choice for monitoring relapse of malignancies. Recently, Nagoshi et al.[73] have followed spermidine and putrescine before, during, and after chemotherapy for hematological malignancies, and concluded that not only spermidine but putrescine and total polyamines are also useful for predicting success or failure of the chemotherapy. The longitudinal studies on malignant lymphomas were also made,[74,75] and showed such usefulness of spermidine as a predictor.

3. *Limitations in Solid Tumors*

The changes in polyamines in body fluids in solid tumors appear generally smaller than in hematological malignancies. Not specific, but all polyamines tend to increase in each kind of solid cancers. It should be pointed out that changes in urinary polyamines in breast cancers are generally small,[76] but those in ovarian[77] and colorectal cancers[7,8] are relatively large. In spite of these relatively small changes of polyamines in solid cancers, their usefulness in monitoring efficacy of therapy and in detecting relapse of cancers was demonstrated for stomach,[7,8,78] colorectal,[7,8] lung,[79] uterine,[77] prostatic,[78,80] and even breast[81] cancers. However, a recent study has shown that predictive values of polyamines are questionable for breast, female genital tract, and prostatic cancers.[82] More detailed data on serial measurements of each polyamine before, during, and after chemotherapy or radiation are required to assess the predictive values of the amines for solid cancers.

B. Acetylpolyamines as Tumor Markers

Acetylpolyamines seem to have opened a new field in clinical aspects of polyamines. The first trial of their assays in cancer patients was made by Tsuji et al.[6] in 1975. Abdel-Monem and Ohno[83] reported that acetylputrescine and N^1-acetylspermidine were excreted into urine of cancer patients in greater quantities than of normal subjects and suggested the usefulness of the ratio of N^1-acetylspermidine to N^8-acetylspermidine. Seiler and Knödgen[60] devised an improved TLC assay for acetylpolyamines and established their normal values in human urine. Abdel-Monem et al.[84] tried to use acetylspermidines for monitoring lymphoma patients and suggested that the N^1/N^8 ratio was useful for prediction of the response to chemotherapy. Mach et al.[85] monitored acetylputrescine and total amounts of acetylspermidines in urine of acute leukemia patients, and concluded that acetylspermidines could be indicators of response to chemotherapy. Nagatsu et al.[86] got higher positive frequency with urinary acetylpolyamines than with hydrolyzed polyamines for various cancers; N^1-acetylspermidine, 70%; N^1/N^8 ratio, 63%; acetylputrescine, 61%; N^8-acetylspermidine, 30%; hydrolyzed putrescine, 66%; and hydrolyzed spermidine, 50%. Therefore, it seems correct that acetylputrescine and N^1-acetylspermidine are comparable or better parameters for cancers as compared with hydrolyzed putrescine and spermidine. In accord with the above results for urine, increased levels of N^1-acetylspermidine were observed in tissues of colorectal adenocarcinoma,[87] breast cancer,[88] and in cyst fluids of malignant brain tumors.[89] However, unfortunately, the levels of acetylputrescine in both normal and cancer tissues are below detection limit. The mechanism of the increase in N^1-acetylspermidine in cancer tissues remains to be elucidated.

C. Materials Used for Analysis

Urine has been widely used for clinical investigation for polyamines, and great numbers of data are accumulated. In most studies 24-hr urine samples were used for polyamine analysis. This is rather laborious for patients and caused an obstacle to make polyamine tests distributed widely. Shinpo et al.[90] studied this problem and concluded that a morning spot urine, if expressed per milligram creatinine, can replace the 24-hr urine without any loss in its value.

In 1979, Takami et al.[91] compared polyamine levels in both plasma and erythrocytes from solid cancer patients and concluded that erythrocyte polyamines can be used as tumor markers; and they gave higher positive frequencies than those in plasma. In the following year, Uehara et al.[92] also reported such utility of erythrocytes. Recently, we have carefully reevaluated human plasma and erythrocytes in clinical investigation for digestive cancer patients.[93] Our results showed that the percent increase of polyamine levels and also positive frequencies in plasma of cancer patients were much higher than those in erythrocytes, in contrast to the report of Takami et al.[91] This is probably due to variable and invariable compartments of polyamines present in erythrocytes; there seem to be relatively fixed polyamine pools in erythrocytes due to ionic binding of spermidine and spermine to their membranes. Thus, a minor percentage of polyamines in erythrocytes varies according to cancer activities as compared with that in plasma. The advantages of the use of erythrocytes are that absolute levels of polyamines in them are much higher than those in plasma, which is more convenient for their analysis by insensitive photometric methods; and that spermidine and spermine in erythrocytes are in unconjugated forms, which requires no hydrolysis procedure prior to assays. Its disadvantage is that polyamine measurements in erythrocytes are more exposed to various artificial factors, because they need washing, isolation, hemolysis, and cell counting. The utility of an assay method seems to exist on the balance between its value of the data obtainable and its simplicity (including rapidness and cost). Thus, whole blood[94,95] rather than isolated erythrocytes should be assessed for polyamine measurements in clinical investigation, because it neither requires creatinine measurement nor is exposed to such artificial factors. This line of experiments is now under way in our laboratories. The utility in predicting the response to chemotherapy or radiation should also be investigated for erythrocytes and whole blood.

The plasma, as mentioned above, seems to be a good material, because it requires no creatinine measurements and its positive frequencies are comparable to those with urine.[93] However, absolute polyamine levels in plasma or serum are much lower than those in urine or erythrocytes, and a considerable percentage of polyamines present in plasma is in conjugated forms, which requires hydrolysis procedure for analysis by conventional methods.

CSF[67,68] and bone marrow aspirates[96] can be used for polyamine measurements. Since the usefulness of polyamine measurements exists in monitoring tumor activities rather than in detecting early cancers, repeated samplings are usually necessary. The repeated samplings of CSF and bone marrow seem too painful for patients. In this respect, urine is best as a material, followed by blood (plasma).

One can easily imagine that gastric juice may be useful for detecting and monitoring gastric cancers. Preliminary studies on this subject were conducted by Kawase et al.,[97,98] but its clinical utility has not been ascertained.

D. Other Factors Affecting Polyamine Levels

The cause of polyamine changes in body fluids is not limited to tumors. Therefore, it is essential to keep in mind various factors affecting polyamine levels for proper assessment of the data obtained in clinical investigation.

1. Nontumor Diseases

Liver cirrhosis[99] and chronic nephritis[100] showed increased polyamines in erythrocytes. Uremia showed increased putrescine, but not spermidine or spermine in serum.[101] All three polyamines were reported to be elevated in cystic fibrosis.[102] Elevated spermidine and spermine were found in the blood of psoriasis patients.[103] Polycythemia vera[62] and sickle cell anemia[104] showed increased levels of the three polyamines in whole blood and in plasma, respectively. Free polyamines in urine were reported to increase in Menkes kinky hair disease.[105] Spermine in blood was reported to increase in systemic lupus erythematosus of infants.[106] However, this should be confirmed by another method because it was conducted by a nonspecific radioimmunoassay. Recently, Womble et al.[107] have reported that urinary polyamines are increased a few days before immunological rejection in heart transplant patients and proposed that the ratio of acetylputrescine to N^1-acetylspermidine is useful for monitoring the clinical course after heart plantation.

2. Physiological Factors

Pregnancy shows increase especially in putrescine in plasma, and spermine in erythrocytes.[108,109] To our knowledge, acetylpolyamines have not been followed during pregnancy.

The daily variation of polyamines and acetylpolyamines in urine or blood was reported by Shinpo et al.[90] and by Hrushesky et al.,[110] respectively. Therefore, it is desirable to fix the time of samplings each day. Shinpo et al.[90] recommended morning spot urine for polyamine measurements.

Blood[111] and CSF[112] polyamines generally decrease with age. The most striking decrease is observed from birth to 1 year for both fluids; and the changes from childhood to adulthood are gradual. Polyamines also tend to decrease during aging.[113]

The excretion of putrescine and spermine into urine in female subjects is higher than in male subjects.[114]

Polyamine excretion is influenced by menstrual cycle. Spermine excretion was 50 times higher in the luteal phase than in other phases,[115] while putrescine and spermidine in urine were highest in the follicular phase.

E. Comparison with Other Tumor Markers

Many tumor markers have been developed.[116] They are divided into two groups, i.e., nonspecific tests for screening and relatively specific markers. The former includes carcinoembryonic antigen (CEA), ferritin, and β_2-microglobulin; the latter includes markers such as α-fetoprotein, thyroglobulin, ribonuclease, and carbohydrate antigen 19-9. Polyamines belong to the former group as tumor markers because of the broadness of their spectrum. Polyamines seem superior to ferritin and comparable to CEA in their specificity and sensitivity.[62,117] Figure 3 shows the test on correlation between polyamines and CEA in various cancer patients; there was almost no correlation between them. Thus, the combination of polyamines and CEA results in better positive frequency (sensitivity) as shown in Figure 4;

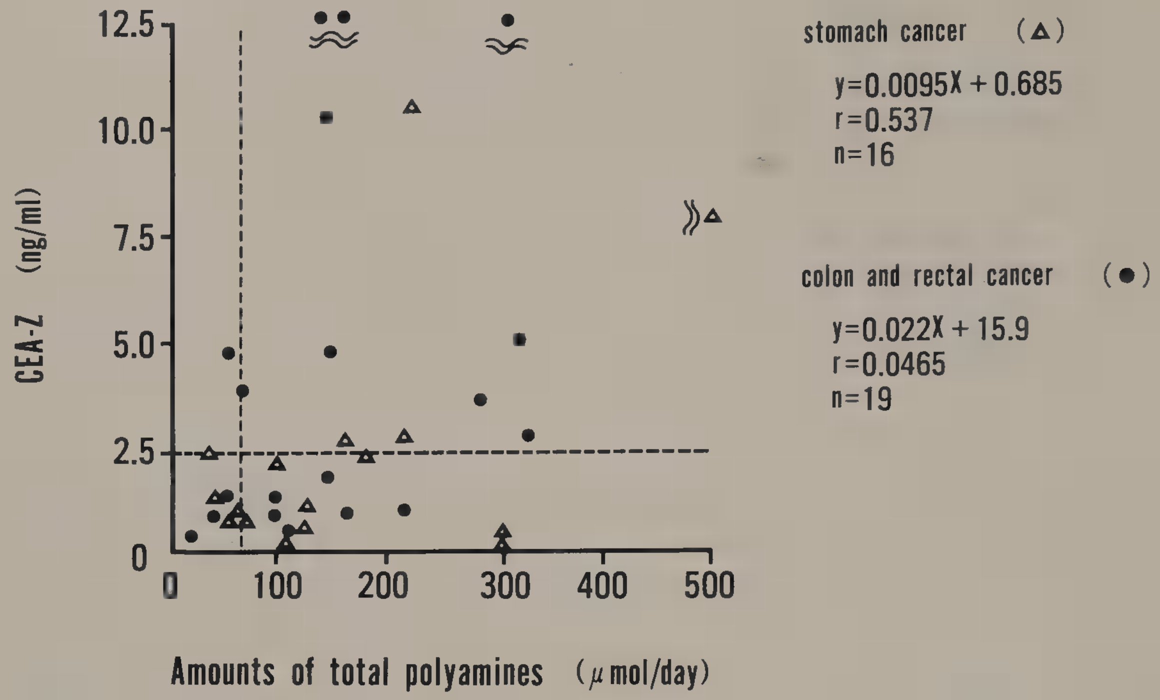

FIGURE 3. Correlation between total polyamines in urine and CEA (Z-gel method) in sera, obtained from stomach and colorectal cancer patients.

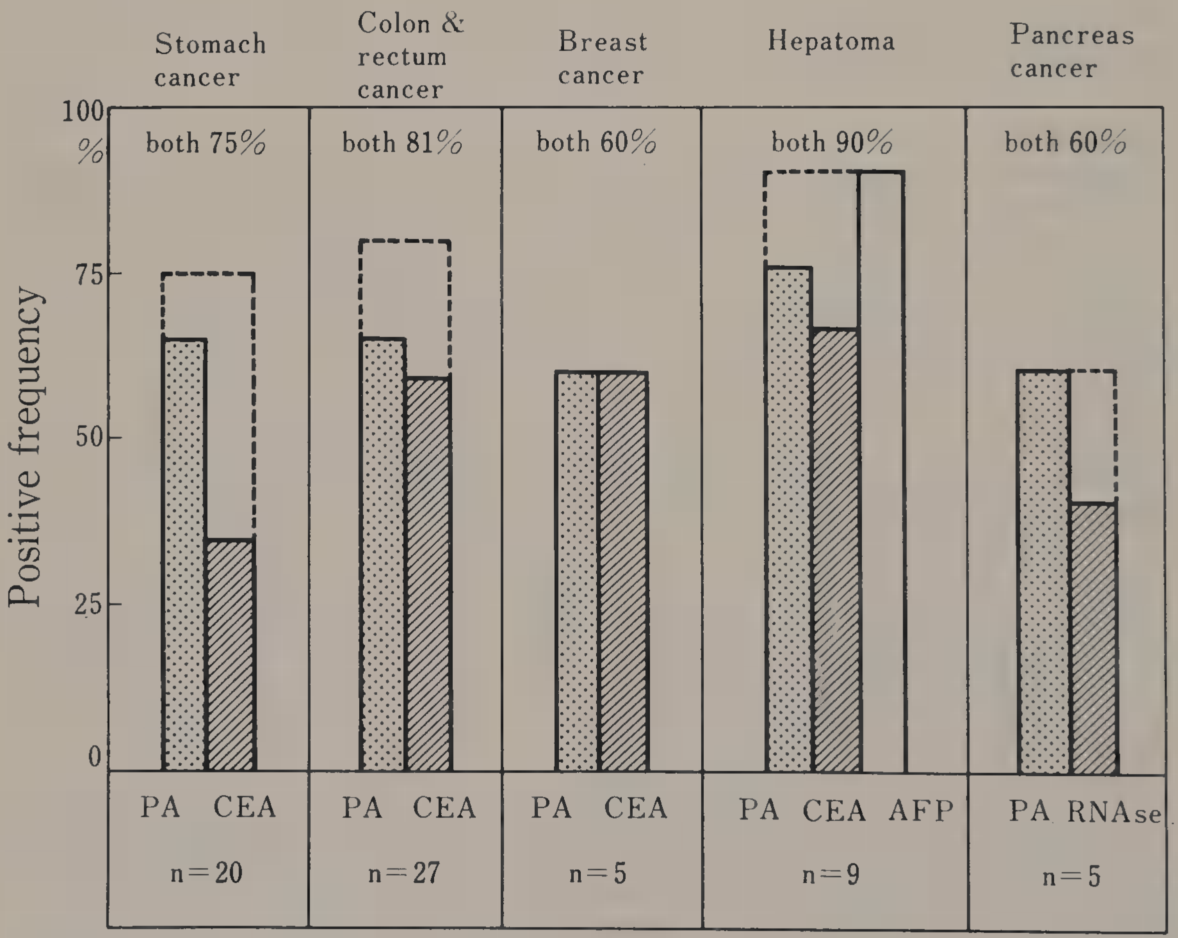

FIGURE 4. Increased positive frequency by combination of polyamines with other tumor markers. PA, polyamine; CEA, carcinoembryonic antigen; AFP, α-fetoprotein; and RNAse, ribonuclease.

but it, of course, causes lower specificity. Therefore, the combined use of polyamines and another sensitive marker is recommended for screening or diagnosis of cancers. At the second step, the relatively specific marker should be employed to eliminate false positive results. Other markers can also be utilized for monitoring tumor activities. However, to predict the response of tumor to chemotherapy or radiation, polyamines seem most useful.

IV. CONCLUSIONS

Evaluations of the clinical utility of polyamines were well made around 1980,[61-65] and the following conclusions were drawn.

1. Polyamines are generally elevated in body fluids according to the activity, metastasis, and stage of a tumor, but not exactly according to tumor size. However, there is poor regularity as to which polyamine increases, especially in solid tumors.
2. Polyamines are disappointing as tumor markers for early diagnosis of cancers.
3. A good correlation is observed between CSF polyamines (especially putrescine) and malignant brain tumors such as medulloblastoma and glioblastoma, and between polyamines in urine or blood and acute hematological malignancies.
4. The sharp rise of polyamines (especially spermidine) observed within 72 hr after chemotherapy predicts tumor response to the therapy, and monitoring of tumor activity with polyamines is useful in clinical investigation.

No major changes were made to these conclusions, and many studies published during the past 5 years have confirmed these ideas. However, we feel it is not necessary to abandon all efforts to utilize polyamines for screening and diagnosis of cancers; there are cancer stages where polyamine tests are positive and it is curable by surgery, chemotherapy, or radiation.

Two points of progress have recently been made in the clinical aspects of polyamines. One sign of progress is the establishment of simple enzymatic assays for total polyamines in urine for routine clinical investigation. Urinary polyamines have now been officially recognized as tumor markers in Japan. Kits for their enzymatic measurements are now commercially available from companies in Japan and Israel. Previously, the most promising method for routine analysis was said to be radioimmunoassays.[55-57] However, this has proved untrue because of the difficulty in preparing antibodies against putrescine and of cross-reaction among polyamines. As stated before, the utility of an assay method exists on the balance between the value of data obtainable and the simplicity (including rapidness and cost) of the method; the routine enzymatic method for urinary polyamines unfortunately includes minicolumn and hydrolysis procedures,[9] which are a little too complicated to be handled in local hospitals. Efforts should be made to make it even simpler and more rapid. An enzymatic method for whole blood polyamines seems to be a promising alternate because it does not require the hydrolysis procedure.

The other progress conducted during the past 5 years is the increased interest in acetylpolyamines as possible tumor markers. The clinical data on acetylpolyamines should further be accumulated to enable us to evaluate their utility. In addition, the mechanisms of the increase in N^1-acetylspermidine in tumor tissues should be investigated. The biosynthesis and transportation of acetylputrescine should also be studied for both normal subjects and cancer patients to explain its very low concentrations in tissues in contrast to high urinary contents.

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Chapter 14

POLYAMINES AS INDICATORS OF DISEASE ACTIVITY AND RESPONSE TO THERAPY IN CANCER PATIENTS

Uriel Bachrach

TABLE OF CONTENTS

I. Introduction 236

II. Polyamines in Biological Fluids 236
  A. Normal Values 237
    1. Effect of Age 237
    2. Polyamines in the Urine of Males and Females 237
    3. Polyamines During the Menstrual Cycle 238
    4. Polyamines in Other Physiological Conditions and Diseases 238

III. Polyamines and Cancer 238
  A. Urine 238
  B. Red Blood Cells 240

IV. Effect of Treatment on Polyamine Levels 241

V. The Use of Polyamines for Staging the Disease and Determination of its Activity 243

VI. Comparison with Other Markers 243

VII. Polyamines as a Tool for the Early Detection of Cancer 243

VIII. Conclusions 244

References 245

I. INTRODUCTION

Any substance which is elevated in tumor patients and correlates with the presence of a tumor may be considered as a biological marker for tumor activity. These markers can be of aid in the diagnosis of malignancy and for screening of the population for the early detection of cancer. They may also be employed for the assessment of the response to antitumor treatment. The early detection of cancer is of great importance. It may increase the chances of the patient to recover, if the treatment is started while the tumor mass is still small. It is also most desirable to have a chemical marker for monitoring the cancer patient undergoing therapy or surgery. Variations in the levels of markers would suggest response to treatment, while unchanged levels would indicate resistance to the therapy used. This approach would be most useful when the estimation of the tumor mass is difficult and when biopsy is not possible. In these cases, the distinction between viable and nondividing tumor cells poses a serious problem. The availability of a reliable monitoring system could lead to a controlled therapeutic approach and could possibly limit the use of excessive irradiation or the administration of unnecessary high doses of toxic antitumor drugs. In addition, follow-up determinations may be useful in appraising relapse or recurrence of disease, prior to the appearance of clinical symptoms.

A perfect tumor marker would be present or be elevated in all tumor-bearing patients and would be absent or be within normal concentration range in all patients without tumors. Unfortunately, no such ideal marker exists.[1] The existing markers are either not general or not elevated in all tumor-bearing patients. Some of them give positive results only with a low proportion of cancer patients. Putrescine, spermidine, and spermine are organic polycations which are involved in many cellular functions.[2-5] Numerous studies have linked these compounds with nucleic acid metabolism, structure, and function, and thus to cellular proliferation.[6] Increased intracellular activity of the biosynthetic enzymes and the accumulation of polyamines indicate active growth.[7,8]

II. POLYAMINES IN BIOLOGICAL FLUIDS

It is now generally accepted that neoplastic growth is associated with the increase in the activity of ornithine decarboxylase (ODC) and with the accumulation of polyamines.[9,10] The apparent increase in ODC activity may be explained by the stabilization of the enzyme, which is expressed by the increase in its half-life.[11] It has also been reported that the rate of ODC synthesis is enhanced in tumor cells.[12] The increase in cellular polyamine levels may lead to the induction of spermidine acetyltransferase (SAT), an enzyme which catalyzes the acetylation of polyamines[13] (but not of diamines). The cytosolic enzyme, thus induced, is responsible for the formation of N^1-acetylspermidine and acetylspermine (see also Volume I, Chapter 11). This acetylation has the following consequences. (1) It leads to the reduction of the net positive charge of the polyamines and thereby reduces their affinity for negatively charged biopolymers. (2) It facilitates a better transport of the polyamines through the cellular membrane. Indeed, N^1-acetylspermidine has been identified in virus-transformed fibroblasts[14] and in lymphocytes transformed by lectins.[15] The activity of SAT also increases in 3T3 NIH cells after transfection with ras-oncogene.[16]

The pioneering studies of Russell[17] showed that diamines and polyamines accumulate in the urine of cancer patients. Moreover, most of these compounds are excreted as acetylderivatives[18-22] and free diamines or polyamines are generated only after acid[23,24] or alkaline[25] hydrolysis. It soon became evident that polyamines released from cancer cells may be detected also in biological fluids other than urine. Extensive studies by Marton and associates[26-31] showed that polyamines and diamines accumulate in the cerebrospinal fluid (CSF) of patients with brain tumors.[32] Subsequent studies showed that the levels of polyamines and diamines are also elevated in the blood of cancer patients,[33-37] mainly in the red blood cells.[35,36]

Since changes in polyamines are noticed in urine, red blood cells, plasma (or serum), and CSF of cancer patients, one wonders which biological specimen should be analyzed for polyamines. It appears that CSF is the best material to be analyzed in patients with brain tumors.[30] According to Marton et al,[29] the best correlations between polyamines and the activity of the disease are obtained in patients with medulloblastoma. On the other hand, repeated sampling of CSF is too painful for patients and the quantities of fluids aspired are limited.

Urine remains the best source for polyamine analyses. Material to be tested may be obtained in large quantities without hurting the patients (see also Chapter 13). The finding that morning urine samples can replace the 24-hr urine pool[38] made the urine analysis less laborious.

Polyamines and diamines are found in the urine mainly as acetylderivatives, while erythrocytes lack these conjugates and contain polyamines and diamines. This may simplify the analytical procedure, but on the other hand, the material is obtained by an invasive approach (for further discussion, see Chapter 13 and Reference 39).

There are conflicting views concerning the utility of polyamine analysis of serum or plasma.[36,40] It is, however, clear that polyamine and diamine levels in plasma or serum are relatively low and that material is obtained by an invasive approach. In addition, hydrolysis of serum (to release polyamines from conjugates), yields amino acids in relatively high amounts. This may pose a serious analytical problem. The relationship between polyamines and neoplastic growth was discussed in several books[6,9] and reviews.[10,24,30,41,42,95]

A. Normal Values

Before attempting to use polyamines as tumor markers, detailed information concerning the normal values should be obtained. The following questions should also be answered:

1. What is the effect of age on polyamine levels?
2. Are there any differences in polyamine levels between males and females?
3. What is the effect of hormones and menstrual cycle on polyamines?
4. Are polyamines elevated also under other physiological conditions or diseases?

1. Effect of Age

It was reasonable to assume that urinary polyamines should be higher in young children then in older ones. This could be related to the growth rates.[43,44] Indeed, young boys and girls excrete more diamines and polyamines than adults.[45-48] Thus diamines and polyamines in the urine of 3- to 4-year-old boys and girls were approximately twice that found in 8- to 11-year-old children.[47] The elevated urinary polyamines may easily be explained by the effect of hormones which are known to stimulate polyamine biosynthesis. According to Rudman et al.,[49] small children, who failed to grow because of deficiencies in growth hormone, had low levels of urinary polyamines and diamines. Administration of growth hormone to these children resulted in an enhanced growth with a concomitant increase in urinary polyamines.[49] Polyamine and diamine levels in the urine of boys and girls were practically identical.[47] There are conflicting views concerning the concentration of polyamines and diamines in red blood cells of children at different ages. According to Casti et al.,[50] blood polyamines decreased with age and changes from childhood to adulthood appeared to be gradual. Other investigators[51] failed to confirm these findings and could not detect significant differences in red cell polyamines of children and adults. Polyamines in the CSF also decrease with age.[52,32]

2. Polyamines in the Urine of Males and Females

Little is known about the levels of polyamines and diamines in the urine of adults. A

Table 1
CONCENTRATIONS OF POLYAMINES AND DIAMINES IN THE URINE OF MEN AND WOMEN OF DIFFERENT AGES[46]

Age group (years)	Sex	Number of samples	Creatinine (mg/mℓ)	Diamines (nmol/mg creatine)	Polyamines (nmol/mg creatine)	Total polyamines
21—30	M	26	2.26 ± 0.103	12.31[a] ± 7.29	5.91 ± 3.14	18.22 ± 7.47
	F	35	1.41 ± 0.072	16.21 ± 5.97	5.17 ± 3.06	21.37 ± 7.41
31—40	M	39	2.13 ± 0.075	10.31[b] ± 4.55	5.55 ± 2.49	15.86[b] ± 5.27
	F	42	1.37 ± 0.073	15.72 ± 5.26	5.09 ± 2.17	20.81 ± 6.09
41—50	M	42	1.73 ± 0.070	11.18[b] ± 6.14	6.10 ± 2.11	17.17 ± 6.25
	F	34	1.39 ± 0.076	15.62 ± 5.73	4.27 ± 2.07	19.89 ± 5.91

[a] $p<0.02$.
[b] $p<0.001$.

recent study[46] revealed significant differences in the levels of diamines in the urine of males and females at the age groups of 31 to 40 and 41 to 50, respectively. Differences between 21- to 30-year-old males and females were less significant (Table 1). These findings are in line with reported data.[53]

3. *Polyamines During the Menstrual Cycle*

Serial studies of whole blood polyamine levels revealed significant differences between males and females.[54] When these blood analyses were followed through the menstrual cycle, definite increases in the spermidine/spermine rates were found at times of known increases in plasma levels of estradiol and progesterone. This corresponds to the known effect of these hormones on polyamine synthesis. (See also Volume I, Chapter 2.) While polyamines and diamines appear to fluctuate in the blood during the menstrual cycle, urinary polyamines and diamines behaved differently.[55] Osterberg et al.[56] measured urinary polyamines in samples collected daily throughout a single menstrual cycle of 13 healthy women. They found that in some samples, polyamine excretion increased during the mid-cycle phase. Recent studies[46] also demonstrated significant variabilities in urinary polyamines and diamines during the menstrual cycle in 4 out of 166 women. It appears that urinary polyamines and diamines are less affected during the menstrual cycle compared to blood polyamines and diamines.

4. *Polyamines in Other Physiological Conditions and Diseases*

Polyamines and diamines are not ideal markers of malignancy, since they lack specificity. It is conceivable that polyamine levels may be elevated during pregnancy due to hormonal effects and the growth of the fetus. Several studies indeed demonstrated an increase in urinary polyamines and diamines in pregnant women. Putrescine and spermine accumulated in the plasma and spermine in the erythrocytes during pregnancy.[9] Table 2 lists some of the other physiological conditions or diseases which give ''false positive results,'' namely increased polyamine levels in biological fluids or cells. For further details, see Chapter 11 by Campbell.

III. POLYAMINES AND CANCER

A. Urine

Numerous studies indicated that the activity of polyamine biosynthetic enzymes is elevated during neoplastic growth and that cancer cells are rich in polyamines.[6,9,41] This phenomenon

Table 2
INCREASED POLYAMINE LEVELS UNDER VARIOUS PHYSIOLOGICAL CONDITIONS AND DISEASES

	Biological sample			
Condition	**Urine**	**Red blood cells**	**Plasma**	**Other**
Pregnancy	Polyamines Diamines[9,85]	Polyamines[57]	Putrescine[58]	Amniotic fluid[57,58]
Liver cirrhosis		Polyamines[59]		
Chronic nephritis		Polyamines[60]		
Uremia			Putrescine[61]	
Gastric ulcer	Polyamines[85]			
Cystic fibrosis		Polyamines[62]		
Psoriasis		Polyamines[63]		Skin, polyamines[64]
Hematological disorders				
Polycythemia vera			Polyamines[65]	
Sickle cell anemia		Polyamines[66]		
Lupus erythematosus		Polyamines[67]		
Surgery	Polyamines[68]		Polyamines[83]	
Jogging			Polyamines[69]	
Insulin-dependent diabetes mellitus	Polyamines[70]			
Muscular dystrophy				Muscle, polyamines[71]
Parasitic diseases		Polyamines Diamines[72]		
Malaria				
Leishmania				Skin, polyamines[73]

appears to be general and was also observed in cells transformed by tumor viruses,[74,75] oncogenes,[16] or carcinogenic compounds.[76] Therefore, it is to be expected that the urine of all cancer patients should contain polyamines and diamines at elevated concentrations. A careful study of the literature reveals that this is not the case and that approximately 80% of cancer patients have high urinary polyamine values (Table 3). Chayen et al.[90] offered an explanation to this observation and suggested that amine and diamine oxidases could be responsible for the reduction of urinary polyamines. This has been substantiated by the oral administration of aminoguanidine to volunteers. This inhibitor caused an increase in urinary polyamines and diamines.[90]

Data depicted in Table 3 suggest that urinary polyamine assays may have clinical utility for monitoring the state of cancer disease. Yet, this notion has not been generally accepted (see also Chapter 12). Thus Horn et al.[91] analyzed 503 urine samples from 192 patients and in 1982 stated that statistically significant differences were found in polyamine levels between patients with active or nonactive disease for tumors of the breast, stomach, prostate, female genital tract, and a variety of metastatic carcinomas of unknown origin. In 1984, Horn et al.[92] reexamined cancer patients and described the results from 938 polyamine determinations. They concluded[92] that for each tumor type, statistically significant group differences were found in polyamine levels between patients with nonactive tumors and patients with active large tumors. On the other hand, they doubted whether polyamine assays could be used to monitor the state of disease for breast tumor. For patients with tumors of the prostate or female genital tract, the results were more promising and elevated levels indicated tumor growth progression in 30 to 50% of the cases.[92]

Seiler et al.[93] studied polyamine excretion in the urine of healthy and tumor-bearing rodents and concluded that urinary polyamine determinations in leukemic mice or tumor-bearing rats have a limited value in the diagnosis of cancer. On the other hand, they observed a significant increase in urinary polyamines 1 day after the administration of adriamycin,[93]

Table 3
POLYAMINES IN THE URINE OF CANCER PATIENTS

Type of cancer	Frequency of elevated polyamines (%)	No. of patients	Ref.
Colorectal	86	7	77
	85	14	78
	70	13	81
	69	32	79
	69	32	80
	66	90	53
	65	52	83
	60	150	85
Stomach	87	8	77
	81	22	78
	78	48	84
	75	94	85
	68	72	79
	68	72	80
Lung	100	4	77
	90	14	86
	80	24	81
	75	102	85
	74	19	84
	67	24	79
	67	24	80
	50	82	53
Breast	85	7	78
	75	115	85
	70	7	25
	50	26	81
	42	14	86
Bladder	100	4	79
	95	18	87
Prostate	70	44	88
Ovary	78	140	85
Nasopharynx	73	30	89
Esophagus	87	8	77
	83	6	84
	83	6	89
Acute leukemia	100	4	24
	100	4	77
	65	14	113
	60	10	81
Hodgkins' lymphoma	100	6	81
Non-Hodgkins' lymphoma	82	162	85
	68	23	81

thus confirming the potential value of polyamines for the assessment of the progress of therapy (see Chapters 12 and 13).

B. Red Blood Cells

It has recently been suggested that determination of polyamines in red blood cells can be clinically useful for diagnosis, evaluation of efficiency of treatment, and follow up of various types of cancer.[36,94-99] There are differences in the opinion whether whole blood or red blood

Table 4
POLYAMINES IN THE RED BLOOD CELLS OF CANCER PATIENTS

Type of cancer	Frequency of elevated polyamines (%)	No. of patients	Ref.
Colorectal	75	24	40
Kidney	81	14	94
Stomach	71	12	40
Bladder	52	47	94
Prostate	63	27	94
Glioblastoma		14	99

cells should be analyzed (see also Chapter 13). Some data summarizing the frequency of elevated polyamines in the red blood cells of cancer patients are given in Table 4 (see also References 9, 24, 42, and 96).

IV. EFFECT OF TREATMENT ON POLYAMINE LEVELS

Urinary polyamine levels in breast cancer are generally low,[92,95] but those in lung,[100] ovaries,[101] and colorectal cancers[77] are relatively high. In spite of relatively small changes of polyamines in solid cancers, their usefulness in monitoring efficacy of therapy has been suggested.[95] Polyamines may be useful for a short-term evaluation of a course of therapy and in long-term evaluation of tumor growth.

The short-term evaluation is based on the finding that polyamine levels in urine and serum increased after successful chemotherapy or radiation therapy.[42] This reflected tumor cell kill. The extent of the elevation was related to the efficacy of the therapy. Thereafter, during tumor regression, polyamine levels returned to normal values. A typical correlation between urinary polyamine levels and the clinical response to cancer therapy was reported by Waalkes et al.[82] Similar results have been reported by others. Table 5 summarizes some of the data indicating the rise of urinary polyamines, within 72 hr after initiating therapy. In all these cases the initial increase in urinary polyamines correlated with a complete or a partial response to therapy. Van den Berg et al.[21] suggested that urine be monitored for polyamine metabolites during the course of therapy and reported that isoputreanine accumulated in the urine as the result of response to therapy.

Regression of the tumor should result in the decrease in urinary or blood polyamines. Table 6 summarizes some of the data and shows that after surgery or successful chemotherapy urinary polyamines returned to the normal values. Similar results were also obtained by Ohsawa et al.[115] who reported that among 30 patients who had elevated urinary polyamines before surgery, 23 patients revealed a decrease to the normal range (77%) after operation. According to Otsuji et al.,[120] urinary polyamines decreased in eight out of ten patients after surgical removal of solid tumors. It is noteworthy that a reduction in red blood cell polyamines was observed in only 40% of 36 patients with solid tumors after curative resection.[120] Kubo et al.[121] also found a decrease in urinary polyamines during the remission period in children. Successful treatment of lymphoma,[113,123] stomach,[79] colon,[79] and brain tumor[124] patients resulted in significant changes in urinary and in red blood cell polyamines.

In the foredescribed studies, polyamines and diamines were determined in urine or CSF samples after hydrolysis. As stated above, acetylputrescine and N^1-acetylspermidine are the major polyamine and diamine conjugates in these biological fluids. It is very likely that these conjugates could serve as better markers of malignancy than polyamines and diamines

Table 5

SIGNIFICANT RISE IN URINARY POLYAMINES WITHIN 72 HR OF INITIATION OF THERAPY CORRELATED WITH A COMPLETE OR PATRIAL RESPONSE TO THERAPY

Disease	No. of patients	Ref.
Acute myelocytic leukemia	56	102
Acute myeloblastic leukemia	12	103
Nonlymphocytic leukemia	7	104
Leukemia, myeloma, lymphoma	42	105
Leukemia	26	106
Leukemia	34	107
Hodkin's	2	23
Lymphoma	39	108
Lymphoma	60	34
Lymphoma	86	114
Non-Hodgkin's lymphoma	20	109
Burkitt's lymphoma	7	82
Hematological and solid tumors	56	113
Multiple myeloma, carcinoma	2	33
Colorectal	53	110
Stomach	12	84
Colon	7	84
Stomach	2	111
Breast	10	112
Ovary	2	85
Lung		115

Table 6

DECREASE IN URINARY POLYAMINES IN RESPONSE TO SURGERY OR THERAPY (LAST 6 YEARS)

Type of cancer	Treatment	No. of patients	Ref.
Breast fibrosarcoma	Surgery	3	89
Larynx	Radiotherapy	3	89
Non Hodgkin's lymphoma	Chemotherapy	20	109
Stomach	Surgery	12	84
Colon	Surgery	7	84
Bladder	Surgery	20	116
Lymphoma	Chemotherapy	21	117
Breast	Chemotherapy	7	118
	Surgery	13	118
Breast	Surgery	6	122
Breast	Chemotherapy	10	112
Stomach	Surgery	9	111
Leukemia	Chemotherapy	12	103
Leukemia	Chemotherapy	10	119

released after hydrolysis. The utility of the determination of acetylpolyamines and diamines for evaluating of long-term courses of therapy is discussed in Chapter 13.

V. THE USE OF POLYAMINES FOR STAGING THE DISEASE AND DETERMINATION OF ITS ACTIVITY

During the past 10 years, significant evidence accumulated that the stage of the disease and the activity of cancer correlate with the concentration of polyamines in biological fluids.

In urine of breast cancer patients, polyamine and diamine levels were higher in Stage III than in Stage I.[85,91] In stomach cancer patients, the frequency of elevated urinary polyamines increased from 11 to 40% at Stage I to 85% at Stage IV.[125,126] Similar differences in urinary polyamines at various stages of cancer of the colon,[127] melanoma,[128] and carcinoma of the prostate[88] were also reported. Polyamine levels in the red blood cells of patients with alimentary tract,[120] brain tumor,[124] or colorectal cancers[129] also correlated with the stage and the activity of the disease. Similar variations were observed when the plasma of colorectal cancer patients were analyzed for polyamines at the various stages of the disease.[126] In uterine cervix cancer patients, diamine and polyamine levels increased in the urine, plasma, and erythrocytes when the disease was more active.[101] All these findings suggest that variations in polyamine and diamine levels in biological fluids could serve as indicators of the clinical status of cancer patients.

VI. COMPARISON WITH OTHER MARKERS

Despite extensive efforts, no reliable specific tumor marker is presently available. Carcinoembryonic antigen (CEA) is among the best tumor markers used clinically to monitor patients with colorectal cancer. This antigen facilitates the detection of a tumor but does not permit the distinction between viable or nonviable cells. Moreover, CEA levels in the blood of cancer patients remains elevated even 14 days after the arrest of tumor growth,[95] unlike polyamines which are indicators of proliferation. In fact, polyamines reflect tumor activity and the arrest in tumor growth will be immediately expressed by a decrease in polyamine levels. Polyamines seem comparable to CEA in sensitivity[125-127] and have the advantage of being a general tumor marker. According to Otsuji et al.,[77] 86 to 100% of urine samples from patients with cancer of the esophagus, stomach, colon, lung, liver, and leukemia had elevated urinary diamines. In the same groups, 25 to 37% of patients with esophagus, stomach, and liver cancers had elevated serum CEA. In colon cancer patients, 71% had elevated CEA (compared with 86% elevated urinary diamines). Half of the lung cancer patients had elevated CEA compared with 100% elevated urinary diamines.[77] None of those patients who had elevated urinary polyamines showed abnormal α-fetoprotein values.[77]

Nagatsu et al.[130] found a good correlation between urinary polyamines and 6-hydroxymethylpterin-like compounds measured by radioimmunoassay. They suggested that the assay of urine for both polyamines (especially putrescine) and 6-hydroxymethylpterin-like compounds may be useful as biochemical markers of cancer.

VII. POLYAMINES AS A TOOL FOR THE EARLY DETECTION OF CANCER

Available data support the notion that polyamines may be used for the assessment of the activity of disease in cancer patients, and for the evaluation of the progress of therapy.[9,95] At present time it is not very likely that polyamines may be employed for the early detection of all types of cancer. The following difficulties should be considered:

1. Wide range of normal values

2. Negative results even in diagnosed cancer patients
3. No changes in polyamines in tumors of small mass and slow proliferation rate

Wide range of normal values — Polyamines and diamines occur in urine, blood, and CSF, and their concentrations vary significantly. It may be inferred from Table 1 that even within a certain age group, significant variations occur. Factors such as diet,[9] environment,[9] stress (see Chapter 11), and menstrual cycle may complicate the picture even more. In women, the variation in blood and urinary polyamines are considerable and there is little chance to detect small variations in polyamine levels. False positive results (Table 2) may obscure the picture, and all the known physiological conditions or syndromes which lead to elevated polyamines should be ruled out.

Negative results in diagnosed cancer patients — False negative results are not rare, even in diagnosed cancer patients who have a large tumor mass with high activity, so that even in these diagnosed cases polyamines cannot be regarded as an absolute tumor marker.

No changes in polyamines in tumors of small mass and slow proliferation rate — Polyamine levels in biological fluids are a function of the proliferation rate of cancer cells or the activity of the disease. It is unlikely that a small tumor mass with slow proliferation rate could be detected by polyamine analyses.

Polyamines may, however, be quite useful to support other tests (histological, enzymatic, or immunological) for the diagnosis of cancer. Polyamines may also be useful for the detection of cancer before the appearance of clinical symptoms if the proliferation rate of the cancer cells is high and the tumor mass is significant. It is also possible that acetylpolyamines could serve as a better marker and could facilitate early detection (see also Chapter 13). Scalabrino (see Chapter 12) also suggested that ODC assays could be used for the identification of cancer cells in biopsy material, so that polyamines and their metabolites or biosynthetic enzyme should be part of the arsenal used in the fight against cancer.

VIII. CONCLUSIONS

Several investigations dealt with the potential clinical value of polyamine measurements in biological fluids. It has been shown that polyamine levels are elevated in patients with different types of cancer. Urine analyses, which were widely used, may be adequate in evaluating tumor status. In many types of cancer, urinary polyamines or diamine levels correlated with the activity of the disease. Urine analyses may have clinical utility in the short-term evaluation of the efficiency of a course of therapy and in long-term evaluation of tumor growth. Plasma (or serum) and erythrocytes have also been analyzed but it is too early to determine whether these materials have any advantage over urine. Polyamine assays in CSF may be useful to obtain clinical information on patients with brain tumors (mainly medulloblastoma).

Polyamines are intimately associated with tumor growth and their measurement provides a good marker of cell kinetics. Other biochemical markers such as CEA have long half-lives and are not as accurate for monitoring disease activity and response to treatment. Most of the other tumor markers are usually specific, unlike polyamines which have the potential of a general marker. On the other hand, the lack of specificity and variability in polyamine levels suggests that their use in cancer screening will be limited. The development of simple, sensitive, and specific methods for the determination of polyamines could facilitate a wider clinical application of this potential tumor marker.

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Chapter 15

INHIBITORS OF POLYAMINE BIOSYNTHESIS AS THERAPEUTIC AGENTS

Juhani Jänne and Leena Alhonen-Hongisto

TABLE OF CONTENTS

I. Introduction 252

II. The Biosynthetic Enzymes of the Polyamines as Targets for Chemotherapy 253
  A. Ornithine Decarboxylase 253
    1. Inhibitors of Ornithine Decarboxylase 253
    2. Antiproliferative Effects of Inhibitors of Ornithine Decarboxylase in Cell Cultures and Tumor-Bearing Animal Models 253
    3. Compensatory Reactions Triggered by Putrescine and Spermidine Depletion and the Development of Resistance to Inhibitors of Ornithine Decarboxylase 255
    4. Combination of Inhibitors of Ornithine Decarboxylase with Cytostatic Drugs 258
    5. Clinical Studies with Inhibitors of Ornithine Decarboxylase 260
      a. Cancer 260
      b. Psoriasis 261
    6. Chemoprevention of Cancer 261
  B. Adenosylmethionine Decarboxylase 262
    1. Inhibitors of Adenosylmethionine Decarboxylase: The Family of Bis(Guanylhydrazones) 262
    2. Structure/Activity Relationship of Bis(Guanylhydrazones) 262
      a. Inhibition of Adenosylmethionine Decarboxylase 262
      b. Inhibition of Diamine Oxidase 263
      c. Cellular Accumulation of Bis(Guanylhydrazones) 264
      d. Antimitochondrial Effects of Bis(Guanylhydrazones) Is There a Role for Carnitine? 265
      e. Effects of Bis(Guanylhydrazones) on Ornithine Decarboxylase and Polyamine Acetylase 267
    3. Antiproliferative Effects of Bis(Guanylhydrazones) in Cultured Cells and in Tumor-Bearing Animal Models 268
      a. Cultured Cells 268
      b. Tumor-Bearing Animals Models 269
      c. Development of Resistance to Bis(Guanylhydrazones) 269
    4. Combination of Inhibitors of Adenosylmethionine Decarboxylase with Inhibitors of Ornithine Decarboxylase and Conventional Cancer Chemotherapeutic Agents 270

            a. Combination with Inhibitors of Ornithine Decarboxylase 270
            b. Combination with Conventional Cancer Chemotherapeutic Agents 272
            c. Polyamine Antimetabolite Combination in Hyperproliferative Skin 272
        5. Clinical Studies with Bis(Guanylhydrazones) 273
    C. Inhibitors of Spermidine and Spermine Synthases 274

III. Concluding Remarks: Is There a Role for Polyamines in Cancer Chemotherapy? 276

Acknowledgments 277

References 277

I. INTRODUCTION

The polyamines putrescine, spermidine, and spermine occur at millimolar concentrations virtually in all living cells. They clearly belong to the ''housekeeping machinery'' of all mammalian cells and seem to be indispensable for normal cellular proliferation to occur. What then is the rationale to approach cancer chemotherapy by preventing the formation of the polyamines? Although there are reports indicating that many tumor cells contain more polyamines and are more actively engaged for their synthesis than untransformed cells, this apparently is not a general rule.[1-3] However, numerous studies with the inhibitors of polyamine biosynthesis (also called polyamine antimetabolites) have shown that these agents are effective cancer chemotherapeutic drugs fully comparable to the conventional cytostatic agents (as reviewed in this chapter). Furthermore, is not dihydrofolate reductase also a housekeeping enzyme, by any definition?

Polyamine biosynthesis in animal cells is accomplished by the concerted action of four enzymes: ornithine decarboxylase (ODC) (catalyzing the formation of putrescine), adenosylmethionine decarboxylase (catalyzing the formation of decarboxylated adenosylmethionine) (AdoMetDC), spermidine synthase (transferring the propylamine moiety from decarboxylated AdoMet to putrescine to form spermidine) and finally spermidine synthase (another propylamine transferase catalyzing the synthesis of spermine from spermidine and decarboxylated Adomet).

From these enzymes the two decarboxylases, ODC and AdoMetDC, are by far the most important targets for a chemical intervention, as they catalyze the rate-controlling reactions in the biosynthesis of the polyamines. The role of the two propylamine transferases, usually occurring in large excess in comparison with the two decarboxylases, as targets for chemotherapy may be less important as almost complete spermidine depletion can be achieved with inhibitors of ODC alone. Furthermore, a depletion of putrescine and spermidine appears to be sufficient to halt proliferation even under conditions where the content of spermine is elevated.[4] It is thus possible that spermine serves as a metabolically dead-end salvage compound that is used for back conversion to spermidine and putrescine under conditions in which the latter polyamines are depleted.

As many aspects of the inhibitors of polyamine biosynthesis have been covered by recent review articles or books,[5-8] the present chapter deals mainly with the therapeutic potential of the antimetabolites of polyamines and especially with the latest advances in this field.

II. THE BIOSYNTHETIC ENZYMES OF THE POLYAMINES AS TARGETS FOR CHEMOTHERAPY

A. Ornithine Decarboxylase

1. Inhibitors of Ornithine Decarboxylase

Inhibition of polyamine biosynthesis serves two purposes. It gives the possibility to elucidate the physiological role of polyamines and offers a means for clinical applications in cancer chemotherapy. Due to its role as a rate-controlling enzyme of the polyamine biosynthesis under most conditions,[1] ODC serves as an obvious target for chemical intervention aimed to produce polyamine depletion.

The early inhibitors of ODC included quite nonspecific pyridoxal 5′-phosphate antagonists[4] and a number of specific substrate and product analogues, which were reversible, competitive inhibitors of the enzyme. Unfortunately, the competitive mode of inhibition makes the compounds useless in vivo as they stabilize ODC and are thus unable to produce a substantial polyamine depletion. Another group of compounds inhibiting putrescine and spermidine synthesis is the family of unphysiologic diamines, such as diaminopropane and 1,3-diamino-2-propanol. The action of diamines does not involve a direct inhibition of ODC but represents some kind of translational repression of the enzyme,[9,10] probably involving a formation of macromolecular inhibitors of ODC.[11] In addition to the possibility that antiproliferative effects of the diamines are not exclusively polyamine related, the use of these compounds is limited also by many other factors to be discussed later.

As regards the specificity, so far the most ideal inhibitors of ODC belong to a group called suicide or mechanism-based enzyme inactivators. The mechanism of action of these drugs, which are congeners of ornithine or putrescine, involves activation by ODC and subsequent irreversible alkylation of the enzyme leading to a rapid loss of activity. Metcalf et al.[12] first reported the synthesis of three such compounds: an ornithine analogue 2-difluoromethylornithine (DFMO) and two putrescine analogues 5-hexyne-1,4-diamine and trans-hex-2-en-5-yne-1,4-diamine. Among these, DFMO has widely been used both in cell cultures and in vivo to produce depletion of putrescine and spermidine.[4] 5-Hexyne-1,4-diamine exerts secondary effects involving GABA metabolism due to its oxidation in vivo to 4-aminohe-5-ynoic acid.[13] To avoid the unwanted effect, modifications of the parent compound were made. A monomethylated analogue of 5-hexyne-1,4-diamine, i.e., (2R,5R)-6-heptyne-2,5-diamine, which is not oxidized by monoamine oxidase, proved to be an extremely potent irreversible inhibitor of ODC. In fact, this compound is at least ten times more potent (on weight basis) than DFMO both in vitro and in vivo.[14] Fluorinated putrescine derivatives, mono- and difluoromethyl putrescine, are likewise potent inhibitors of ODC but share several properties of putrescine that make them less suitable for in vivo use.[15] α-Allenyl derivatives of putrescine have no advantage over DFMO as inhibitors of mammalian ODC, although they may have potential as inhibitors of bacterial polyamine biosynthesis.[16]

2. Antiproliferative Effects of Inhibitors of Ornithine Decarboxylase in Cell Cultures and Tumor-Bearing Animal Models

The effect of inhibition of ODC by different types of inhibitors has been reviewed in detail by Heby and Jänne.[4] In general, depletion of intracellular putrescine and spermidine contents results in growth inhibition reversible by the addition of exogenous polyamines. Only recent studies dealing with DFMO or other irreversible inhibitors with cancer chemotherapeutic potential will be discussed here.

Medrano et al.[17] reported a differential effect of DFMO on the proliferation of untransformed and chemically transformed cells. The growth-inhibited untransformed cells resumed growth upon removal of DFMO while transformed cells did not unless exogenous polyamine was added. In contrast to virally transformed cells, which under condition of polyamine depletion stop their growth at S phase of the cell cycle,[18] the chemically transformed cells as well as their untransformed counterparts appeared to accumulate in the same phase of the cell cycle.[17] The authors suggested that the observed difference in the effect of DFMO on proliferation was related to a selective inhibition of the synthesis of specific proteins, one of them possibly being ODC itself.

The specificity of polyamine requirement for L1210 leukemia cell proliferation in the presence of DFMO has been studied with structural homologues of spermidine and putrescine.[19,20] In addition to the data of structural requirements for prevention of DFMO-induced cytostasis in L1210 cells, the studies also revealed that the critical levels of polyamines for cell proliferation were 30% of normal spermidine and 60% of spermine. The fact that treatment of cells with DFMO alone seldom decreases the intracellular levels of spermine, has been considered to be responsible for the cytostatic rather than cytotoxic action of the drug.

Complete growth arrest and cytotoxic effect with DFMO has been achieved in few cell lines.[21-23] In the case of human lung carcinoma cells, the cytotoxic effect of DFMO is related to anchorage-independent type of cell growth.[24] Moreover, comparison of small cell lung carcinoma, towards which DFMO acts cytotoxically, with nonsmall cell lines with cytostatic response to DFMO revealed that ODC activity was 25 times lower in the small cell than the nonsmall cell cultures.[25] Moreover, levels of ODC mRNA were 100-fold lower in the small cell that the nonsmall cell line indicating a close relationship between DFMO sensitivity and steady-state levels of ODC mRNA. Treatment of rat hepatoma cells with (2R,5R)-6-heptyne-2,5-diamine resulted in a depletion of putrescine, spermidine, and also spermine leading progressively to growth arrest and ultimately to cell death.[26] When compared with DFMO, part of the better efficacy of this putrescine analogue in cell culture may be attributable to its more efficient intracellular accumulation.[26]

Pera et al.[27] compared the effects of four irreversible inhibitors of ODC in two cell lines with different ability to metabolize 5′-methylthioadenosine, a by-product of polyamine synthesis. The cells with a specific 5′-methylthioadenosine phosphorylase were more sensitive to the inhibitors DFMO, 2-(fluoromethyl)dehydroornithine, 2-(fluoromethyl)dehydroornithine methyl ester, and (2R,5R)-6-heptyne-2,5-diamine; otherwise the different polyamine responses were similar in the two cell lines. Among the four compounds, (2R,5R)-6-heptyne-2,5-diamine was the most effective inhibitor of both polyamine synthesis and cell proliferation.[27]

There are several systems relating ODC induction and growth stimulation by hormones. Inhibition of polyamine biosynthesis with DFMO blocked the growth-promoting effect of 17β-estradiol in *N*-methyl-*N*-nitrosourea-induced rat mammary tumor grown in soft agar[28] or in hormone-responsive human breast cancer cells[29-31] indicating that polyamines are essential for hormone-mediated mitogenic effect on breast cancer cells. Recently polyamines were shown to be necessary for the autocrine control of tumor growth by the hormone.[32] In addition to polyamines, extrinsic serum and pituitary factors were shown to potentiate the mitogenic effect of estradiol on human breast cells.[32] Proliferation of prolactin-dependent lymphoma cells represents another system of hormone-controlled growth.[33] Long-term suppression of ODC activity with DFMO inhibited lactogen-stimulated growth, while prolactin-independent cell variants were less sensitive to DFMO indicating that loss of hormonal dependence may be associated with increased resistance to DFMO.[33] Androgen response in mouse kidney has been shown to include induction of ODC and subsequent accumulation of putrescine.[34] The finding that inhibition of ODC and depletion of putrescine by DFMO did not abolish kidney cell hypertrophy in response to testosterone[35] is contradictory to earlier conclusions that putrescine might be a mediator of androgen response in mouse kidney.[36]

The antiproliferative effect of DFMO in the treatment of experimental tumors has been shown in a numer of studies.[37] DFMO reduced growth of renal adenocarcinoma, increased survival of tumor-bearing mice, comparable to the antitumor effect in mouse L1210 leukemia,[38] and prevented lung metastases.[39] Similarly, DFMO decreased the secondary pulmonary metastases in mice with Lewis tumor.[40] However, DFMO did not affect the growth of another renal tumor, Wilms' tumor, even though putrescine and spermidine levels were significantly depleted.[39] The authors suggested that Wilms' tumor cells may be able to alter their requirements for polyamines during the course of their growth.

The successful treatment of human small cell lung carcinoma in culture with DFMO naturally led to in vivo experiments in which athymic mice were implanted with cultured human small cell tumor. Treatment of mice with DFMO inhibited tumor growth and lengthened survival of animals, especially of those with low tumor burden.[41] Long-term maintenance therapy with cyclic DFMO administration was designed to avoid host toxicity of DFMO such as weight loss and dose-limiting thrombocytopenia observed during continuous administration.[42] Growth inhibition of lung tumor implants could be maintained for over 1 year in mice, suggesting that this kind of DFMO treatment could be employed in maintaining clinical remission. Intravenously administered DFMO inhibited growth of methylcholanthrene-induced sarcoma in rats in a dose-related fashion.[43] Although the higher doses used caused side effects such as platelet suppression and shortening of the villi in the duodenum and jejunum, the lowest effective dose (500 mg/kg/day) was without side effects.[43]

The effect of DFMO on the host defense system in mice bearing tumor allografts has been demonstrated to be selective, modulating cytotoxic T-lymphocyte and antibody-mediated responses, but not affecting nonspecific host defense mechanisms, natural killer-cell activity, and phagocytic activity.[44] Specific response against tumor challenge was initially delayed but its period as a whole was prolonged.

The usefulness of (2R,5R)-6-heptyne-2,5-diamine as an antitumor drug has been shown by Bartholeyns et al.[45] in a study in which treatment with the drug reduced growth of EMT6 sarcoma and HTC hepatoma in vitro, prolonged survival of mice bearing L1210 or P388 leukemias, and inhibited the development of Lewis carcinoma. Superiority over DFMO was demonstrated by the fact that in each case the effective doses of (2R,5R)-6-heptyne-2,5-diamine were 10- to 20-fold lower than those of DFMO and that at these antitumor doses given chronically no apparent host toxicity was observed. Thus, taken together all the properties of the drug, (2R,5R)-heptyne-2,5-diamine appears to be a promising antitumor drug.

3. Compensatory Reactions Triggered by Putrescine and Spermidine Depletion and the Development of Resistance to Inhibitors of Ornithine Decarboxylase

All inhibitors of ODC induce secondary effects that diminish their efficacy to produce polyamine depletion. Some of these unwanted effects result from a direct action of the drugs on the biosynthetic enzymes of polyamines such as stabilization of ODC by competitive inhibitors, activation of AdoMet decarboxylase (DC) by certain diamine derivatives in the absence of putrescine or serving as substrates of propylamine transferases leading to formation of analogues of higher polyamines capable of supporting growth.[4] In addition to the direct effects, drug-induced polyamine depletion appears to trigger a variety of secondary compensatory reactions aimed to conserve the intracellular polyamine pools required for continuous proliferation.

Cells depleted of their putrescine and spermidine by 2-methylornithine or DFMO effectively maintained their intracellular polyamine concentrations by diminishing the excretion of polyamines[46,47] or by increasing the uptake of the natural polyamines from extracellular medium.[48,49] Diamine inhibitors, however, greatly enhanced the excretion of especially spermidine indicating that diamines may release polyamines from their intracellular binding sites.[47] Polyamine-depleted cells effectively accumulated polyamines also in vivo.[50] The fact that not only the natural polyamines but also the unphysiological amines or compounds closely related to the polyamines are taken up at an enhanced rate by DFMO-treated cells was used as a means to replace the natural polyamines by cadaverine and its aminopropyl derivatives in cultured Ehrlich ascites cells.[51] Under certain conditions, polyamine-depleted or starved cells appear to be able to synthesize cadaverine through decarboxylation of lysine by ODC.[52-54]

Treatment of mammalian cells with inhibitors of ODC resulted in an induction of AdoMetDC that was repressible by polyamines.[55,56] As a consequence, the content of decarboxylated AdoMet was greatly increased in polyamine-depleted cells due to the absence of the natural substrates for aminopropyltransferases that use decarboxylated AdoMet as an aminopropyl donor.[57,58] It is thus possible that some effects of DFMO are attributable to the effects of decarboxylated AdoMet on cellular methylation or on adenine nucleotide pools. Unexpectedly though, DFMO treatment rather increased than decreased total cellular adenine nucleotide pools.[59,60] Pegg et al.[61] recently described the acetylation of AdoMet in vitro by nuclear acetyltransferases and in SV-3T3 cells exposed to DFMO suggesting a novel metabolic fate for decarboxylated AdoMet. Interestingly, decarboxylated AdoMet was shown to be an inhibitor of histone acetylation, a phenomenon which may contribute to the secondary effects of DFMO in vivo.[61]

Already early experiments with 2-MO or DFMO showed that cells exposed to the drugs for extended periods of time gradually developed resistance to the drugs. Mamont et al.[62] first reported the selection of a rat hepatoma cell clone partially resistant to the antiproliferative effects of 2-MO and DFMO. These cells showed elevated activity of ODC suggesting an overproduction of the enzyme. Ehrlich ascites carcinoma cells continuously grown in the presence of cadaverine and DFMO likewise exhibited exceptionally high activity of ODC.[51] Direct evidence for overproduction of ODC in DFMO-resistant CHO cells or mouse lymphoma cells was provided by Choi and Scheffler[63] and by McConlogue and Coffino.[64,65] The latter authors also suggested, based on morphological appearance of chromosomes, that amplification of ODC genes was involved in overproduction of ODC mRNA and protein. The availability of cloned cDNAs encoding ODC[66-68] has enabled the quantitation of ODC gene dosage and amount of ODC mRNA in tumor cells resistant to high concentrations of DFMO. Southern blot analyses revealed several signals for ODC genes in wild-type mouse lymphoma and myeloma cells but only one of them was amplified in DFMO-resistant cells.[66,67] Apart from gene amplification ODC overproducers also expressed the message of the enzyme at an enhanced rate. Ehrlich ascites carcinoma cells resistant to very high concentrations of DFMO appeared to contain multiple amplified ODC genes.[69] The resistant Ehrlich ascites cells, when grown in the absence of DFMO, only slowly returned to the normal level of gene dosage indicating a stable chromosomal amplification. Phenotypic changes seen in cells with amplified ODC genes and in the revertant cells may be related to the tumorigenicity of these cells,[70] although a direct causal relationship has not been established. A single-step selection of DFMO-resistant L1210 cells in the presence of cadaverine resulted in overproduction of ODC based almost exclusively to enhanced accumulation of ODC mRNA with only a modest degree of gene amplification (1 to 2 copies) as shown in Figure 1.[71] In L1210 cells, the mechanism of the development of drug resistance appeared to involve greatly enhanced transcription of ODC mRNA prior to a possible gene amplification. McConlogue et al.[72] described a similar phenomenon in a series of mouse S49 cell variants selected for resistance to 0.1 m*M* DFMO by a single or a multistep process. In these variants, overproduction was based on increased steady-state levels of ODC mRNA and in some variants the translability of ODC mRNA was enhanced, but none of the variants showed a real ODC gene amplification.[72]

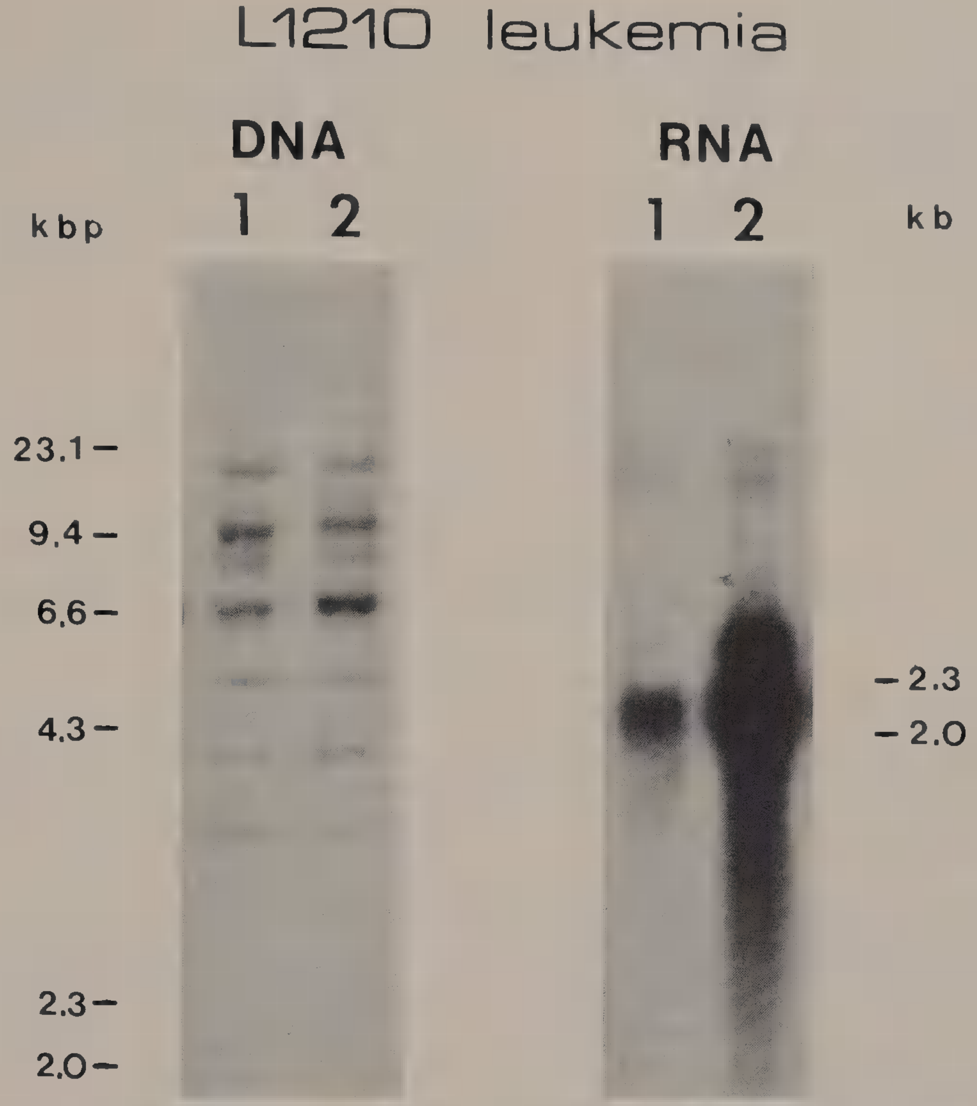

FIGURE 1. Restriction analysis of ODC sequences in genomic DNA and amounts of ODC mRNA isolated from L1210 leukemia cells. DNA and RNA were isolated from parental L1210 cells (lane 1) or from cells exposed to 10 m*M* DFMO and 5 μ*M* cadaverine (lane 2) for more than 1 year. It is noteworthy that the gene dosage and the accumulation of RNA in overproducer cells have remained unchanged during a period of about 1 year, i.e., since the first published results[71] despite the continuous selection pressure.

L1210 leukemia cells selected for DFMO resistance in culture can maintain this resistance also in vivo, as illustrated in Figure 2. Survival of mice inoculated with parental L1210 cells was significantly lengthened by administration of DFMO (panel A), whereas DFMO exhibited no antileukemic effect in mice inoculated with ODC-overproducer L1210 cells (panel B). This finding suggests that the easily developing resistance to DFMO may render tumors unresponsive to treatment with DFMO also in a clinical setting.

We recently described the selection of the first human cell line with amplified ODC gene(s).[73] As their murine counterparts, the DFMO-resistant Sultan human myeloma cells contained elevated amounts of ODC mRNA and overproduced ODC protein. A further characterization of these cells revealed that the sequences of ODC genes segregate between chromosomes 2 and 7,[74] the sequences in the short arm of chromosome 2 being amplified. Interestingly, N-*myc* oncogene having the same subchromosomal localization was not coamplified with the sequences of ODC in the Sultan cells.[75] On the other hand, a number of human tumors (neuroblastomas) that exhibited amplification of N-*myc* gene did not show any amplification of ODC genes.[74]

Cells with DFMO-resistant phenotypes were recently selected after growth for 72 hr in

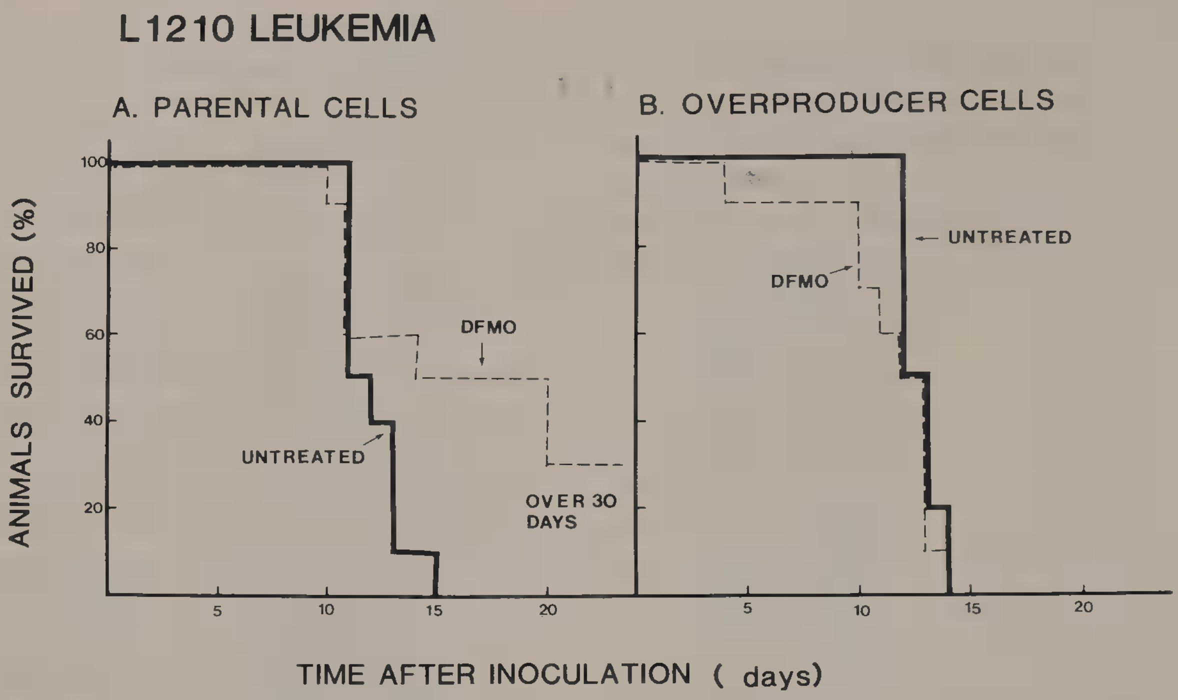

FIGURE 2. Effect of DFMO on survival of mice bearing L1210 leukemia. The DBA/2J mice were inoculated with cultures parental L1210 cells (panel A) or with ODC overproducer cells grown in the presence of DFMO and cadaverine (panel B). The DFMO-treated mice received 2% DFMO in drinking water during the entire period of the experiment.

the presence of low concentration of cycloheximide.[76] These cells exhibited low ODC activity, no ODC gene amplification, and unaltered DFMO transport, as judged by the ability of the drug to cause polyamine depletion. It is suggested that cycloheximide induced the expression of some genes, other than ODC, advantageous for proliferation in the absence of putrescine and spermidine.[76] Such growth-supporting changes could include cycloheximide-mediated superinduction of c-*myc* mRNA[77,78] or c-*fos* mRNA.[79]

4. Combination of Inhibitors of Ornithine Decarboxylase With Cytostatic Drugs

With all the DFMO-induced compensatory mechanisms in mind, it is obvious that treatment with DFMO alone is not sufficient to produce total polyamine depletion, but for this purpose DFMO should be combined with inhibitors intervening other reactions of polyamine biosynthesis. When cytotoxic effect is desired, irrespective of polyamine depletion, DFMO, being itself mainly cytostatic, may be used to modify the effects of drugs with established or potential chemotherapeutic efficacy. Combination of DFMO with antineoplastic drugs has been recently reviewed in detail by Porter and Jänne.[80]

Pretreatment with DFMO modified the response of 9L rat brain tumor cells to DNA crosslinking agents and intercalators. The cells were sensitized by DFMO-treatment to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) both in culture and in vivo[81,82] providing that the cells originally were sensitive to BCNU.[83] It has been proposed that DFMO-induced polyamine depletion, by altering DNA conformation, influences chromosomal damage and crosslink formation by the cytotoxic drug.[84-86] This is in line with the fact that chromosomal damage, measured as sister chromatide exchanges, and crosslink formation produced by BCNU was increased by DFMO-pretreatment, whereas the effects of *cis*-diaminechloroplatinum (II) on DNA were decreased in polyamine-depleted cells[85] together with the decreased cytotoxicity in 9L cells.[87] The enhancement of BCNU-induced cytotoxicity has been confirmed in L1210 cells[88] and in human adenocarcinoma cells.[89] However, in contrast to the antagonistic effect in 9L cells, the combination of DFMO and *cis*-diaminedichloroplatinum (II) has been shown to produce an additive or synergistic effect in human and hamster pancreatic cancer cell lines[90] and in P3J, a Burkitt's lymphoma cell line.[91] Controversial results have also been obtained with an antineoplastic DNA intercalator, adriamycin. The cytocidal effect of adriamycin in human adenocarcinoma cells was reduced by DFMO pretreatment[92] although the combination of DFMO and adriamycin was synergistically effective in P3J cells[91] and prolonged the survival of tumor-bearing mice and rats.[93] Instead of DFMO, (2R,5R)-6-heptyne-2,5-diamine combined with adriamycin produced at least additive antitumor effect in vivo.[45] These controversies may be attributable to the tumor type-specific effects of the combinations.

DNA crosslinkers of various types have been combined with DFMO in the treatment of cultured cells. The cytotoxicity of aziridinylbenzoquinone was decreased by polyamine depletion in 9L cells[94] while the combination of DFMO and L-phenylalanine mustard in a human lymphoma cell line showed increased toxicity with increased number of crosslinks, partly due to a delay in the removal of crosslinks.[95] Partial polyamine depletion produced by DFMO decreased the sensitivity of three human tumor cell lines to chlorambucil but had no effect on cell survival after thiotepa treatment.[96] The differing response of DFMO-treated cells to DNA crosslinkers possibly depends on the type and mechanism of formation of crosslinks.

Cell death in response to X-irradiation involves damage to cellular DNA. Survival of 9L rat brain tumor cells after X-irradiation was not influenced by depletion of polyamines by DFMO[97] although polyamine depletion decreased the sensitivity of 9L cell DNA to X-irradiation suggesting an alteration in DNA conformation.[84] A recent study demonstrated that DFMO-induced polyamine depletion served as a radiosensitizing pretreatment in human breast carcinoma cells and inhibited the repair of radiation-induced potentially lethal damage.[98] The finding that the maximum radiosensitization was achieved at 10 m*M* DFMO concentration after 1 hr incubation, which was not sufficient to maximally inhibit ODC, may be taken as an evidence that polyamine depletion alone was not responsible for the observed effects.

The specific effect of DFMO on the cell cycle traverse of certain tumor cells has laid basis on the combined use of DFMO and cell cycle-specific cytotoxic drugs. DFMO-treated HeLa cells accumulated in S phase of the cell cycle being thus sensitive to S phase-specific arabinosyl cytosine (Ara-C).[99] More generally, selective killing of tranformed cells by a combination of DFMO and Ara-C was achieved because transformed cells were found to be accumulated in S phase by DFMO treatment whereas their normal counterparts were protected by being accumulated in G1 phase.[100] The combination was effective also in vivo leading to synergistic increase in the survival of mice bearing L1210 leukemia.[101] DFMO likewise sensitized transformed or tumor cells to other S phase-specific agents hydroxyurea[102] and 5-fluorouracil.[103] Unfortunately, this kind of selective killing of tumor cells is hampered by the fact that all tumor cells do not respond to DFMO in the same way. DFMO may slow down the cell cycle traverse in general or cause accumulation in other phases than S phase.[104,105] A probable reason for DFMO-induced decreased cytotoxicity of Ara-C, methotrexate, and mitosis-specific vincristine in 9L rat brain tumor cells is overall inhibition of cell cycle traverse the DFMO allowing less cells to enter the vulnerable phase of the cell cycle.[106,107] Nevertheless, combination of DFMO with vindesine increased the therapeutic effect of vindesine on animal tumors[93] and combination of DFMO and vinblastine sulfate had an additive effect on the growth of a human cell line in culture.[91]

The enhanced uptake of extracellular polyamines and related compounds by polyamine-depleted cells may be used for transportation of cytotoxic polyamine derivatives into cells. Probably based on this mechanism, the cytotoxic effect of aziridinyl putrescine in a prostate-derived tumor cell line was greatly potentiated by DFMO pretreatment.[108] Several N^4-spermidine derivatives have also been tested for their antiproliferative effect in DFMO pretreated cells.[109] The latter study suggested potential of spermidine derivatives to serve as vectors for biologically active moieties. The rationale behind and the actual use of the combination of DFMO and MGBG will be discussed in detail later.

Promising results have been obtained with combinations of DFMO and biological modifiers. DFMO in combination of mouse type I interferon almost totally suppressed the growth of B16 melanoma, a tumor type which is very sensitive to DFMO alone, both in culture and in mice.[110] The finding that interferons inhibit ODC activity[111,112] may contribute to the observed efficacy of the combination of interferon with DFMO, although this combination depleted melanoma tissue polyamines to the same extent as DFMO alone.[110] Sunkara et al. reported potentiation of the antitumor and antimetastatic effect of DFMO with interferon inducers against B16 melanoma and Lewis lung tumor in mice suggesting that the combination could enhance both immune response or interferon-mediated cytotoxicity.[113] Combination of human recombinant interferon with DFMO was beneficial in the treatment of human tumor cells in culture[114] and in nude mice.[115] Modulation of malignant growth may also be brought about by vitamins or hormone analogues combined with DFMO. In a recent study, the four-agent combination of DFMO, dexamethasone, interferon, and retinoic acid suppressed melanoma growth at concentrations at which single drugs were ineffective.[116] Significant loss of efficacy was observed if DFMO was left out or putrescine added indicating that the growth-suppressing properties at least partly depended on an inhibition of polyamine synthesis.

As already mentioned earlier, polyamines may be involved in mediating the effect of estrogens on rat mammary tumors. DFMO has been combined with an antiestrogen tamoxifen to inhibit the growth of *N*-nitrosomethyl-urea-induced mammary tumors in soft agar or in rats.[30,117-119] In these systems, DFMO blocked the mitogenic effect of estradiol but was not found to potentiate the antitumor effect of tamoxifen. Furthermore, exogenous polyamines reversed the cytostatic effect of tamoxifen alone suggesting that tamoxifen may act by a polyamine-mediated mechanism.[30,118]

5. *Clinical Studies with Inhibitors of Ornithine Decarboxylase*

a. Cancer

Although the chemotherapeutic effect of DFMO as a single agent in the treatment of patients with cancer has not been as good as expected on the basis of its antitumor effect in numerous animal studies,[120] it is probable that many human tumors with high levels of ODC and polyamines[121] would serve as suitable targets for DFMO therapy. DFMO is well tolerated, and the toxic side effects, such as dose-limiting thrombocytopenia, anemia, and hearing loss, are transient, disappearing when the treatment is discontinued.[120] Gastrointestinal toxicity is reduced by using i.v. instead of oral administration.

Despite the results from a phase I study indicating that steady-state drug levels during the treatment with the oral DFMO dose of 2.25 to 3.00 g/m^2 every 6 hr should be sufficient for antitumor activity,[122] the subsequent trials have failed to demonstrate an antitumor effect in cancer patients. The efficacy and toxicity of DFMO in the treatment of advanced small cell lung cancer and metastatic colon cancer has recently been evaluated in a phase II trial.[123] Among the 23 patients with lung cancer, only one partial response was observed. Patients with colon cancer showed no responses. The positive finding was that the latter patients, who received DFMO on a schedule of 3 weeks on, 1 week off, had significantly less side effects, including lack of thrombocytopenia.[123]

Since the effect of DFMO generally is only cytostatic, it may be reasonable to combine DFMO with other clinically useful drugs. An example of combining polyamine antimetabolites was the successful treatment of childhood leukemia with DFMO and MGBG.[124] A recent phase I study showed synergistic clinical activity of the combination of DFMO and interferon in melanoma patients.[125] Partial remissions were observed in 2 of 12 patients with metastatic melanoma, and minor responses in two additional patients. This combination will be further tested in a phase II study.

b. Psoriasis

Psoriasis is a nonmalignant skin disease characterized by hyperproliferative epidermal growth with increased accumulation of polyamines. Experimental studies using various models of mouse skin have shown that DFMO, administered topically or systemically, prevented polyamine accumulation and subsequently inhibited tissue growth indicating the potential use of DFMO in the treatment of hyperproliferative disorders of human skin.

Topical application of 2-methylornithine on psoriatic lesions did not result in reduction of skin polyamine concentrations and the clinical improvement was minimal.[126] However, seven of ten patients treated topically with DFMO were moderately improved. In these patients DFMO significantly reduced putrescine and spermidine concentrations of the skin.[126] In another clinical trial, the effect of anthralin was compared to that of DFMO.[127] With anthralin, concentrations of all three polyamines were reduced and the epidermal thickness of psoriatic skin was decreased, while DFMO, despite putrescine and spermidine depletion, had no effect on clinical status of psoriatic skin.[127]

Because of the marginal clinical efficacy of DFMO treatment, partly attributable to poor permeability into human skin, it is obvious that DFMO has not gained a foothold in the treatment of psoriasis. It is, however, possible to combine DFMO with conventional treatment regimens of psoriasis, such as retinoids, dithranol, or PUVA. No reports of such combinations in clinical trials are yet available.

6. Chemoprevention of Cancer

Chemoprevention of cancer is a mode of clinical therapy in which occurrence of cancer is prevented with the aid of chemical agents. Chemopreventive agents may inhibit any of the multiple steps of carcinogenesis, such as formation of carcinogens or interaction between carcinogens and cellular target sites, or suppress neoplastic cellular manifestations when administered after exposure to carcinogens.[128] Among the inhibitors of ODC, DFMO with its relatively low toxicity and known mechanism of action makes a potential candidate for a chemopreventive agent irrespective of the scarce knowledge of the role of ODC induction in carcinogenesis. In fact, DFMO has been shown to be inhibitory in several models of chemical carcinogenesis, including retardation of promotor-induced tumor formation in epidermal two-stage carcinogenesis.[129,130] DFMO also inhibits growth of tumor implants in animals as reviewed earlier. These results suggest that DFMO could be used as a chemopropylactic agent in the treatment of patients with high risk of cancer or in maintenance therapy after remission to prevent recurrence of tumors.

DFMO reduced the incidence of colonic tumors in mice treated with 1,2-dimethylhydrazine as well as reversed preneoplastic changes in colonic mucosa.[131] Moreover, DFMO inhibited increase of colonic ODC activity and potentiation of carcinogenesis in rats by promoters of human colorectal cancer.[132] The situation of animals with colon tumors resembles that of patients with familial polyposis in that the colonic polyps from these patients have increased ODC activity.[133] It has thus been suggested that chronic treatment with DFMO could prevent polyp formation and neoplasia. The preventive treatment would be especially useful in young unaffected first-degree relatives who can be expected to develop polyps.[121]

Administered in urine, DFMO inhibited tumor promotion initiated by *N*-methyl-*N*-nitrosourea in heterotopically transplanted rat urinary bladders.[134] This study has led to a suggestion that DFMO could be used clinically to prevent the frequent recurrence of human bladder cancers after surgical resection.

The incidence of mammary tumors in mice and rats after treatment with a carcinogen has been reduced by DFMO.[135-137] In rat mammary carcinogenesis, administration of DFMO in drinking water as a 0.125 to 0.5% solution seemed to be sufficient to produce therapeutic effect.[137] Tamoxifen treatment of mice bearing *N*-methyl-*N*-nitrosourea-induced breast tumors induced tumor regression both in the presence and in the absence of DFMO.[119] In this system, DFMO did not increase the efficacy of tamoxifen in inducing tumor regression, although the combination of suboptimal doses of both drugs inhibited ODC activity and lowered tumor spermidine content.[119] Nevertheless, the available experimental data support the idea of using DFMO as a chemopreventive agent to inhibit the initial occurrence and recurrence of breast cancer in humans.

Contradictory results to the potentially beneficial effect of DFMO in carcinogenesis have been reported by Kamatani et al.[138] They showed that polyamine depletion produced by DFMO increased spontaneous mutation frequency in rat basophilic leukemia cells treated with *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine. It is thus possible that, under certain conditions, depletion of cellular polyamine levels may increase the rate of carcinogenesis in which spontaneous mutation frequence may play an important role.

B. Adenosylmethionine Decarboxylase

1. *Inhibitors of Adenosylmethionine Decarboxylase: The Family of Bis(Guanylhydrazones)*

Since the discovery of Williams-Ashman and Schenone[139] indicating that methylglyoxal bis(guanylhydrazone) (MGBG) is an extremely potent inhibitor of eukaryotic, putrescine-activated AdoMetDC, this compound has served as the standard inhibitor of polyamine biosynthesis and as a model for further chemical design. Although approaches have been made to develop inhibitors of AdoMetDC on the basis of nucleotides related to AdoMet and decarboxylated AdoMet,[140] MGBG and its congeners remain by far the most important group of AdoMetDC inhibitors. There are two main reasons for this conclusion. First, among the bis(guanylhydrazones) are the most potent inhibitors of the enzyme,[8] and second, these compounds possess concrete therapeutic potentials. Moreover, most of the bis(guanylhydrazones) when combined with inhibitors of ODC produce a synergistic anti-proliferative effect (see Section B.4).

Although probably hundreds of bis(guanylhydrazones) have been synthesized[141] since the discovery of the strong antileukemic activity of MGBG and the parent compound glyoxal bis(guanylhydrazone) (GBG),[142] these compounds have only been screened for possible growth-inhibitor activity. It was only a few years ago, when the interest in bis(guanylhydrazones) was renewed and the group is under reevaluation, but now as inhibitors of polyamine biosynthesis. The new screening principle has to take into consideration not only the inhibition of AdoMetDC, but also other activities exerted by the bis(guanylhydrazones). These include their cellular accumulation, inhibition of other enzyme activities, most notably that of diamine oxidase, the suitability for combined use with inhibitors of ODC and effects not related to the inhibition of spermidine and spermine formation, such as antimitochondrial effect. These properties will be dealt with in detail below.

2. *Structure/Activity Relationship of Bis(Guanylhydrazones)*

a. Inhibition of Adenosylmethionine Decarboxylase

Although MGBG is an extremely potent (Ki value around 200 n*M*)[8] inhibitor of AdoMetDC, it is not the most effective compound in this respect among the bis(guanylhydrazones). Corti et al.[143] already found that further alkylation of the glyoxal portion of the molecule, such as in ethylglyoxal bis(guanylhydrazone) (EGBG), resulted in a more profound inhibition of AdoMetDC than that exerted by MGBG. The fact that increased hydrophobicity of the glyoxal portion enhanced the inhibitory action towards AdoMetDC was later documented and extended by others.[8,140,144] In fact, the most potent inhibitor of the enzyme so far described is apparently ethylmethylglyoxal bis(guanylhydrazone) (EMGBG), exhibiting an apparent Ki value of only 12 n*M*.[145] Replacement of the methyl group of MGBG by a propyl group (PGBG) seems already to decrease the affinity for the enzyme.[146] In addition to the possibly too bulky alkyl group, the decreased affinity towards the enzyme may also contribute the fact that the C–N=N–C groupings of PGBG, unlike in MGBG, are not totally planar but slightly tilted.[147]

French et al.[148] were the first to point out that there is a striking resemblance between the structures of MGBG (or GBG) and spermidine. This resemblance may have something to do with the mechanism of AdoMetDC inhibition by MGBG. Already Williams-Ashman and Schenone[139] noticed that the inhibition of AdoMetDC by MGBG was much more pronounced in the presence of putrescine, the activator of the enzyme, than in the absence of the diamine. Furthermore, MGBG much more profoundly inhibited putrescine-activated AdoMetDC from ventral prostate and baker's yeast than the magnesium-activated enzyme from *Escherichia coli*.[139] In line with these observations was the finding of Hölttä et al.[149] indicating that MGBG apparently inhibits AdoMetDC uncompetitively in respect to putrescine. In other words, MGBG binds to an enzyme form that cannot any more bind putrescine, i.e., to an enzyme complex already containing bound putrescine. The same authors[149] reported that MGBG acts as a competitive inhibitor in respect to AdoMet, the substrate of the reaction. At the first sight, this may not make sense, as there apparently is not much in common between an aliphatic bis(guanylhydrazone) and AdoMet that is a nucleoside derivative. The fact, however, remains that all the bis(guanylderivatives) so far tested, i.e., GBG, MGBG, EGBG, PGBG,[8] and EMGBG[145] show straightforward competitive inhibition pattern with respect to AdoMetDC. Feasible or not, Porter and Dave[150] proposed that the spacing of various carbon and nitrogen atoms and the location of the primary amino groups in the MGBG molecule had almost perfect match to those in AdoMet. Interestingly, also the sulfonium-attached methyl group in the AdoMet molecule has its properly spaced counterpart in the MGBG molecule. Indirect evidence to support the view that bis(guanylhydrazones) really compete with AdoMet is derived from the fact that the affinity of the parent compound GBG, which lacks the methyl groups just mentioned, is not more than one tenth of that of MGBG.[151]

All the bis(guanylhydrazones) mentioned here are reversible inhibitors of AdoMetDC and, although experimentally only documented in the case of MGBG,[4] all the compounds supposedly stabilize otherwise labile AdoMetDC enzyme against intracellular degradation, a phenomenon essentially weakening the enzyme inhibition under conditions in vivo.

Some attempts have been made to develop irreversible inhibitors for AdoMetDC among the derivatives of bis(guanylhydrazones). The first of these was a compound called MBAG, a close congener of MGBG, having the primary amino groups of the latter compound replaced by hydrazine groups.[152,153] The proposed irreversible mode of action, in comparison with MGBG, of MBAG may not be that straightforward one.[154] In any event, this compound has not been used in recent years in vivo, probably because of its overt toxicity and it apparently does not have any therapeutic potential. More recently, Karvonen et al.[155] reported that two old antiparasitic drugs, namely Berenil and pentamidine, which are aromatic congeners of bis(guanylhydrazones), irreversibly inhibited eukaryotic AdoMetDC. However, the apparent affinities for the enzyme were orders of magnitute lower than those of best aliphatic bis(guanylhydrazones). Moreover, no data were given as regards their usefulness as inhibitors of polyamine accumulation. Accordingly, it is impossible to assess the therapeutic potential of such compounds, as this (see below) requires much more extensive comparative studies with MGBG also including all the unwanted effects exerted by the latter drug.

b. Inhibition of Diamine Oxidase

As mentioned above, the characterization of the inhibition of AdoMetDC by compounds related to MGBG is not enough to assess the therapeutic potential of the substance as a polyamine anitmetabolite. Possessing two elements of aminoguanidine, a classic inhibitor of diamine oxidase, it was not totally unexpected that MGBG was found to be a potent inhibitor of diamine oxidase (DAO).[149,156] The practical consequences, as regards polyamine depletion, was first pointed out by Hölttä et al.[149] who found that injections of near toxic doses of MGBG did not reduce spermidine and spermine contents in rat thymus. This phenomenon was clearly attributable to the powerful inhibition of the exceptionally high thymic diamine oxidase activity.[149]

Of great practical and even clinical significance is the fact that the highest DAO activity in mammals including man[157] resides in the intestinal epithelium, probably functioning as a protective means against bacterial-derived and dietary diamines and polyamines. When administered in vivo, MGBG almost totally blocks the oxidative degradation of putrescine for long periods of time.[156] In this respect, the irreversible inhibitor MBAG is even more powerful.[156] It has likewise been shown directly that therapeutic doses of MGBG not only virtually abolish any DAO activity in mouse intestine in vivo[158] but also strikingly promote the transfer of intestinal diamines into systemic circulation.[159] Although in some tissues (such as thymus, see above) exhibiting high DAO activity, the inhibition by MGBG of the latter enzyme already is the reason for failures to achieve effective polyamine depletion, this phenomenon (inhibition of DAO by MGBG) has its most important impact during treatments with combined DFMO and MGBG, as described in Section B.5.

As outlined above, the blockage of DAO activity by any inhibitors of AdoMetDC is a central issue not only because of any unknown general consequences resulting from this inhibition but also in terms of spermidine and spermine depletion achievable by the depression of decarboxylation of AdoMet. During our screening of the members of the bis(guanylhydrazones), we have systemically measured the ratio of AdoMetDC inhibition to the inhibition of DAO exerted by any of the test compounds. Initial studies[144] already indicated that the alkylation of the GBG molecule (such as in EGBG) not only improved the inhibitory potency towards AdoMetDC, but also made the compound less effective as regards the inhibition of DAO activity. GBG has a Ki value of 6 μM for AdoMetDC and 0.2 μM for DAO. Already MGBG, i.e., one extra methylene group, is much better in this respect as it is equally effective towards both enzymes (Ki values about 200 nM).[8] EGBG is even better in this respect, the ratio of the Ki values of AdoMetDC inhibition to that of DAO is 1 to 10, i.e., the drug inhibits AdoMetDC activity at concentration of one tenth of those required for the inhibition of DAO activity.[8,144] Again, the ethylmethyl derivative (EMGBG) seems to be the most suitable inhibitor of AdoMetDC as it is comparatively weak inhibitor of DAO activity.[145]

Out of the known irreversible bis(guanylhydrazone) inhibitors, MBAG is even more powerful inhibitor of DAO activity than MGBG producing a long-lasting blockage of putrescine oxidation also in vivo.[156] It has been recently reported that phenyl bis(guanylhydrazone) is a powerful inhibitor of DAO (Ki value at micromolar range) whereas double phenylated bis(guanylhydrazone) was practically inactive.[160] Nothing is known about the effects of these compounds on the activity of eukaryotic AdoMetDC.

The inhibition of DAO activity by inhibitors of AdoMetDC is important from the point of view of achieving effective polyamine depletion, however, in terms of cytotoxicity the cellular accumulation of the compounds is of utmost importance.

c. *Cellular Accumulation of Bis(Guanylhydrazones)*

As already mentioned, probably hundreds of bis(guanylhydrazones) were screened in late 1950s and early 1960s for their antileukemic activity,[141] but only a few of them were found to exert growth-inhibitory effect comparable to that of MGBG.[142] The compounds classified as inactive ones also included EGBG[161] which is a much better inhibitor of AdoMetDC than either GBG or MGBG, as we know today. Adamson et al.[161] were the first to point out that the inactivity of EGBG was attributable to the fact that L1210 mouse leukemia cells accumulated three times as much MGBG as the inactive EGBG. Although the exact mechanism whereby bis(guanylhydrazones) enter the living cells is not known, it is generally believed that these compounds utilize the putative polyamine carrier. Based on competition studies with radiolabeled spermidine. Dave and Caballes[162] proposed that MGBG utilizes same transport system (facilitated diffusion) as does spermidine. The system probably is linked to sodium transport and is utilized by a variety of amines and related compounds.[163] Although the transport system for the natural polyamines and for the bis(guanylhydrazones) generally is poorly characterized, there is one feature of the system, which clearly possesses therapeutic impact: the rate of drug/polyamine transport is linearly related to the cellular growth rate.[164,165] In other words, rapidly dividing cells accumulate MGBG and also its more alkylated derivatives[144] much faster than quiescent cells do. We will return to this point again when describing the antitumor spectrum of MGBG.

It has been increasingly evident that it is not the influx component of the transport system but rather the efflux component that determines the efficacy of the intracellular accumulation of the bis(guanylhydrazones).[8] Alhonen-Hongisto et al.[166] showed that although the apparent affinities for the putative polyamine carrier (as determined by short-term competition experiments with radiolabeled spermidine) of GBG, MGBG, and EGBG were only marginally different, a long-term accumulation of GBG and MGBG were about five times more efficient than that of EGBG. In subsequent efflux studies[166] it became evident that while GBG and MGBG were effectively retained by tumor cells, EGBG was to a large extent excreted in a few hours. In line with the above experimental results was the finding of Wiseman et al.[167] who described a human variant cell line resistant to MGBG. In short-term uptake studies these cells took up the drug at seemingly normal rate, but again released the drug at strikingly enhanced rate in comparison with the parental cell line.[167]

One of the striking features of the MGBG transport system is the fact that an exposure of tumor cells to MGBG for short periods of time appears to result in a stimulation of the uptake of the natural polyamines and MGBG itself.[168] This phenomenon, when combined with the profound inhibition of DAO activity (see Section b), may have practical consequences as regards the failure to achieve reductions of the polyamine pools during treatments with MGBG in vivo.[168]

Putrescine and spermidine depletion produced by inhibitors of ODC strikingly enhance the accumulation not only of the natural polyamines but also that of MGBG[48] and GBG,[151] but not that of EGBG[169] or PGBG.[146] This phenomenon, i.e., the fact that a priming with DFMO greatly enhances the accumulation of MGBG, led to the sequential combination with DFMO and MGBG in the treatment of experimental and human cancer (see section 4).

Among the bis(guanylhydrazones) tested, the accumulation and retention of GBG appears to be most efficient,[166] and also indusive by a prior exposure to DFMO.[151] Unfortunately, GBG is the weakest inhibitor of AdoMetDC activity. It is thus obvious that the ideal bis(guanylhydrazone) remains to be discovered.

d. Antimitochondrial Effects of Bis(Guanylhydrazones): Is There a Role For Carnitine?

Although MGBG is an extremely potent inhibitor of AdoMet DC and hence prevents the formation of spermidine and spermine, it has been known for a long time, that this drug also is a mitochondrial poison.[150,170] This effect, although it may be connected to the intracellular functions of the natural polyamines, is certainly not related to the inhibition of AdoMetDC. MGBG induces in cultured cells overt morphological changes in mitochondria, including swelling and disappearance of the cristae.[150] Most of the studies regarding the mechanistic implications of the antimitochondrial action of bis(guanylhydrazones) have been performed using isolated mitochondria. Bis(guanylhydrazones) appear to compete with the natural polyamines for binding sites at the outer surface of the inner mitochondrial membrane.[171] The drugs likewise inhibit mitochondrial respiration[172] and mitochondrial DNA synthesis.[173] In 1979 Mikles-Robertson et al.[174] reported that the antimitochondrial effect exerted by MGBG was strictly dependent on the proliferative activity of the cells, i.e., rapidly dividing cells exhibited earlier mitochondrial damage when exposed to MGBG. This phenomenon is in all likelihood attributable to the fact that rapidly proliferating cells accumulated the drug much faster than quiescent cells.[165] The latter authors also noticed that nondividing lymphocytes were resistant to MGBG but when stimulated by lectin showed profound drug-induced mitochondrial damage. However, it is known that lectin-induced stimulation of lymphocytes is associated with a more than tenfold increase in the accumulation of MGBG.[144] In any event, the antimitochondrial effect may be responsible for the toxicity of the drug directed to the epithelial linings of the gastrointestinal tract.[175,176]

In addition to MGBG, out of the bis(guanylhydrazones) only EGBG has been tested for its antimitochondrial action in animal cells. The interpretation of the results of these studies is somewhat problematic as high intracellular concentrations of EGBG are difficult to achieve. Seppänen et al.[169] reported that L1210 leukemia cells exposed to 5 μM EGBG for 24 hr did not show any pathological changes in mitochondrial morphology in contrast to cells exposed to 5 μM MGBG for only 12 hr. However, a determination of intracellular drug levels indicated that the concentration of EGBG was only one third of that of MGBG under these conditions.[169] In support of the mitochondrial sparing effect of EGBG, in comparison with MGBG, were experiments indicating that MGBG was more effective in releasing spermidine and spermine from isolated mitochondria in vitro.[169] Similar difficulties are encountered when interpretating the results of Igarashi et al.[177] Again EGBG, unlike MGBG, was reported not to induce mitochondrial damage or depression of mitochondrial pyruvate oxidation; however, the apparent concentration of EGBG was only about 20% of that of MGBG.[177]

Our unpublished observations have indicated that GBG likewise damages mitochondria in cultured L1210 cells, yet the damage is morphologically distinct from that induced by MGBG.

Related to the antimitochondrial activity of MGBG we found[178] that the drug-inhibited carnitine-dependent oxidation of long-chain fatty acids in muscle homogenates at concentrations readily achievable in vivo. The inhibition was competitive in respect to carnitine and occurred also in cultured L1210 leukemia cells exposed to 5 μM MGBG.[178] D,L-Carnitine distinctly prevented the early mitochondrial damage of cultured L1210 cells exposed to MGBG without affecting the cellular accumulation of the latter drug.[179] However, there seemed to be a threshold concentration of MGBG above which the protective effect of carnitine disappeared.[179] Contradictory to the view that the growth-inhibitory effect exerted by MGBG would be based on the early mitochondrial damage was the finding indicating that in the presence of mitochondria-protective concentrations of D,L-carnitine MGBG fully retained its antiproliferative activity in cultured L1210 leukemia cells (our own unpublished experiments).

We have recently extended these studies to ascertain whether carnitine also would protect experimental animals from MGBG-induced toxicity and death and ultimately whether carnitine could serve as a means to broaden the otherwise narrow therapeutic index of MGBG. As shown in Figure 3, D,L-carnitine indeed protected both NMRI (panels A and B) and DBA (panel C) mice from acute deaths induced by very high single doses of MGBG. The doses of MGBG were as follows: panel A, 275 mg/kg; panel B, 200 mg/kg; and panel C, 200 mg/kg, i.e., more than twice the LD50 dose given by Mihich.[141] We also tried to protect tumor-bearing animals (Ehrlich ascites carcinoma and L1210 leukemia) with carnitine in the connection of high-dose MGBG treatment. In case of Ehrlich ascites carcinoma there was an only marginal therapeutic effect with the carnitine administration[246] and none in case of mouse L1210 leukemia.[247] It is, however, possible that only large, systematic dose-finding (as regards both MGBG and carnitine) studies will reveal whether the therapeutic range of MGBG can be broadened with the aid of a concomitant carnitine administration.

Although the antimitochondrial effects exerted by the bis(guanylhydrazones) are distinctly not related to an inhibition of AdoMetDC, they may be related to the binding and function of the polyamines in the mitochondria.

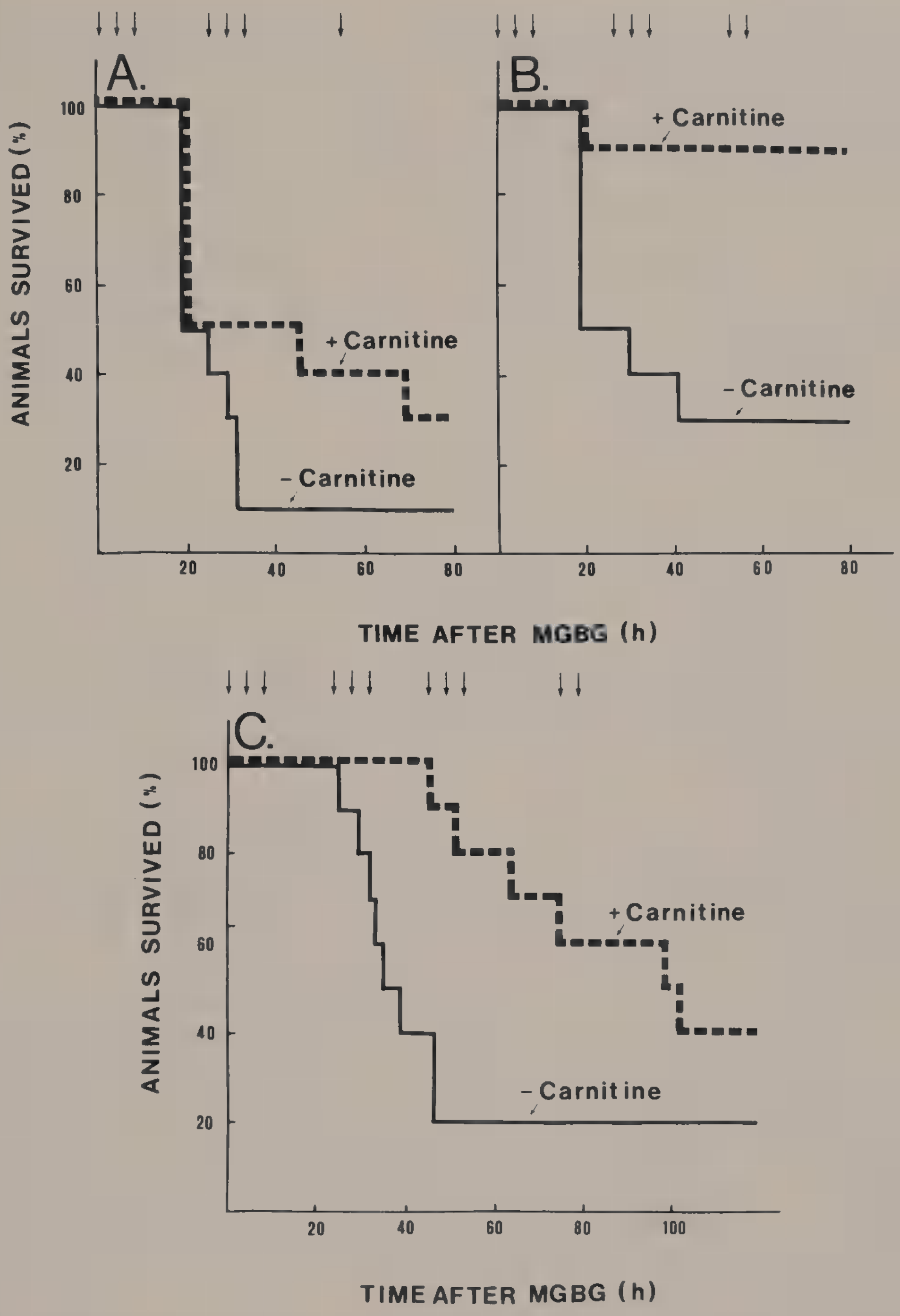

FIGURE 3. Effect on carnitine on the toxicity of MGBG in mice. Female NMRI mice (ten in each group) received a single injection of 275 mg/kg (panel A) or 200 mg/kg (panel B) or female DBA mice (ten in each group) received 200 mg/kg (panel C) or MGBG at time point 0. The arrows indicate the administration of DL-carnitine, the first injection being 1000 mg/kg and the subsequent injections 400 mg/kg.

e. Effects of Bis(guanylhydrazones) on Ornithine Decarboxylase and Polyamine Acetylase

Administration of therapeutic doses of MGBG into rats[149] or mice[180,181] resulted in a distinct stimulation of ODC activity which is at least partially attributable to a stabilization (by unknown mechanism) of the enzyme.[182] Out of the bis(guanylhydrazones) tested MGBG and EGBG enhanced the enzyme activity and the accumulation of immunoreactive protein whereas GBG inhibited both the activity and the accumulation of the enzyme protein.[183] None of the drugs had any effect on the accumulation of mRNA of ODC.[183] The fact that MGBG and EGBG were better stabilizers of ODC than GBG was did not explain the differential effects exerted by the bis(guanylhydrazones) on ODC accumulation, as also GBG distinctly stabilized the enzyme.[183] Calculations of the rate constants for enzyme synthesis and degradation revealed that GBG, and possibly also MGBG, acted as apparent translational inhibitors of ODC synthesis.[183]

Just like ODC, also the activity of spermidine/spermine acetyltransferase is stimulated upon exposure to MGBG.[182]

3. Antiproliferative Effects of Bis(Guanylhydrazones) in Cultured Cells and in Tumor-Bearing Animal Models

As much of the literature concerning MGBG is covered by recent extensive review articles or book chapters[5-8] only new developments and changing views will be reviewed here.

a. Cultured Cells

Under most cell cultures MGBG exerts a clear-cut antiproliferative effect at micromolar (1 to 10 μM) concentrations.[4-7] The antiproliferative effect is most profound in cells with high proliferative activity. This phenomenon is obviously attributable to the fact that the accumulation of the drug is critically dependent on growth rate[165] and since millimolar (assuming even distribution) drug concentrations are needed for cell kill.[184] It is also obvious that the growth inhibition is more closely related to intracellular drug levels than to the spermidine/spermine depletion achieved.[184] Animal cells concentrate MGBG were effectively so that large concentration gradients are rapidly established across the cell membrane. Cell growth begins to slow down at an intracellular concentrations of about 0.5 to 1 mM or 100 to 200 μmol/cell.[164,184,185]

Cultured cells can be rescued from MGBG toxicity by an addition of the natural polyamines (putrescine, spermidine, and spermine), 3,3′-iminobispropylamine at micromolar concentrations or microgram ions at millimolar concentration.[163] Interestingly, closely related diamines, 1,3-diaminopropane, 1,3-diamino-2-propanol, cadaverine, and 1,6-diaminohexane appear to be inactive in competing for the cellular uptake of MGBG.[184]

As mentioned earlier, most of the more (than MGBG) alkylated bis(guanylhydrazones) (such as EGBG) were reported to be devoid of antileukemic activity in the early screening studies.[141] However, reevaluation of these compounds in cultured tumor cells have revealed that at least EGBG acts antiproliferatively. In fact, in L1210 mouse leukemia cells the latter compound, at equimolar concentration, was almost as effective as MGBG in slowing down the growth of the cells.[169] EGBG likewise inhibited the accumulation of spermidine and spermine at much lower intracellular concentrations than MGBG did, yet at the same time it gave rise to a more massive putrescine accumulation than resulted from an exposure to similar concentrations of MGBG.[169]

Igarashi et al.[177] compared EGBG and MGBG in concanavalin A-activated bovine lymphocytes. They also found that EGBG effectively inhibited spermidine and spermine accumulation at intracellular concentrations of only one fifth of the effective concentrations of MGBG. Under these conditions, EGBG likewise inhibited DNA and protein synthesis, yet less effectively than did MGBG.[177,186] In these studies, EGBG unlike MGBG, did not interfere with the integrity or function of mitochondria at concentrations sufficient to produce growth inhibition[169] or depression of macromolecular synthesis.[177] It is thus possible that bis(guanylhydrazones) can inhibit cellular growth without producing mitochondrial damage.

The double alkylated (ethylmethyl) derivative of GBG, i.e., EMGBG, did not inhibit the growth of cultured L1210 cells at concentrations from 5 to 200 μM,[145] an observation which is in agreement with the only written account of EMGBG.[187]

In contrast to the alkylated derivatives, the parent compound GBG was shown to be active against L1210 leukemia in vivo already in the primary reports on the carcinostatic activity of bis(guanylhydrazones).[142,148] This compound in cultured L1210 cells mainly lowers the concentration of spermidine, but in contrast to EGBG and MGBG, without concomitant putrescine accumulation.[151] Similarly, in mouse melanoma cells GBG effectively decreased the concentration of spermidine that of spermine remaining unaltered, and again, in contrast to MGBG and EGBG, without any putrescine accumulation.[188] GBG acts antiproliferatively in both experimental systsms (L1210 leukemia and mouse melanoma), yet probably being slightly inferior to MGBG in this respect.[151,188] In comparison with MGBG, GBG appeared to be more effective in depressing thymidine incorporation but less potent in inhibiting leucine incorporation than MGBG.[151]

Although cell culture studies with various bis(guanylhydrazones) may be directly applicable to studies in tumor-bearing animals, this is not always the case. It must be strongly pointed out that many of the disturbing environmental factors, such as high DAO activity and the possibility to take up extracellular polyamines are not existing under most cell culture conditions. The differences between cells growing in culture and cells transplanted into living animals are most striking when combined regimens with DFMO and MGBG are employed (see Section 4).

b. Tumor-Bearing Animal Models

In contrast to the cell culture work, much of which has been performed in 1970s and 1980s, the studies aimed to the elucidation of the spectrum of experimental tumors sensitive and insensitive to bis(guanylhydrazones) (= MGBG) were, in essence, already completed in 1965.[141,189] The picture that emerged at that time was clear enough: with one or two exceptions, all the tumors sensitive to MGBG were experimental leukemias or lymphomas.[141,189] In fact, in their first report on the carcinostatic activity of MGBG (and GBG), Freedlander and French[142] used L1210 mouse leukemia as their experimental model. These early studies clearly revealed that with the possible exception of ascitic (but not solid) Sarcoma 180, all sensitive experimental tumors were leukemias.[141] Thus the experimental data generated 20 years ago clearly indicated that MGBG would not be effective against solid tumors, as recently also shown by Williams and Murphy[190] in their studies with transplantable murine renal cell carcinoma. Together with this ineffectiveness against experimental solid tumors and the fact that for an efficient accumulation of the drug, and hence for its cytotoxicity a rapid growth rate of the tumor cells is required, one wonders the rationale for the embarkation for large clinical trial program in late 1970s (see Section 5.b) almost exclusively involving slowly growing solid tumors. Purely from the experimental point of view, such a program was doomed to fail.

Already the early experiments indicated that the antileukemic activity of MGBG was fully comparable to the conventional antimetabolic cytostatics such as methotrexate, 6-mercaptopurine, 5-fluorouracil, etc.[141] However, its therapeutic range appeared to be extremely narrow. This is exemplified by the fact that in mice the ratio between LD90 and LD10 for MGBG was only 1.3, the same ratio for antimetabolites being something between 1.8 and 2.3.[141]

From the other bis(guanylhydrazones) only GBG has been used in a limited number of experiments with tumor-bearing animal models. In fact, in the connection of the first report on the antileukemic activity of MGBG,[142,148] the parent compound GBG was shown to be roughly as active as MGBG.

The animal toxicity of MGBG includes antiproliferative toxicity, cardiotoxicity, hepatotoxicity, and irreversible hypoglycemia in several species.[150,189] However, the toxicity of the drug, especially in rodents, is only of limited value to predict human toxicity as MGBG is rapidly excreted in rodents without any signs of cumulative toxicity.[141,189]

c. Development of Resistance to Bis(Guanylhydrazones)

Freedlander and French[142] already observed that tumor cells may rapidly become resistant to GBG or MGBG. Resistant strains were crossresistant to both bis(guanylhydrazones) but not resistant to methotrexate, mercaptopurine, or azaguanine.[142] In most cases described so far, the resistance towards MGBG appears to be attributable to an impaired accumulation of the drug in the tumor cells. Adamson et al.[161] described a variant L1210 cell line that was resistant to MGBG. Based on kinetic studies with radioactive MGBG they reached the conclusion that the poor accumulation of the drug in the resistant cells was based, not on an impaired uptake, but on an enhanced release of the drug from the cells.[161] However, the latter authors also described another variant cell line resistant to MGBG in which the loss of sensitivity to the drug was based both on diminished permeability, as well as on decreased binding capacity. Mandel and Flintoff[191] isolated Chinese hamster ovary cells and rat myoblasts resistant to MGBG by a single-step selection. They found that the uptake of the polyamines by the resistant cells was less than 1% of that by the wild-type cells. The stability of the resistant phenotype and its enhancement by mutagenesis led them to conclude that the phenotype has a genetic origin. Interestingly, the resistant cells did not show any alterations in AdoMetDC activity.[191] Gaugas and Chu[192] identified a subpopulation of rat T-lymphocytes highly resistant to MGBG. No information is available about the nature of this resistance. As already mentioned (Section 2.c), Wiseman et al.[167] recently isolated a human variant cell line remarkably resistant to MGBG and subsequently showed that these cells poorly accumulated the drug due to enhanced release from the cells.

Thus the development of resistance to bis(guanylhydrazones) clearly differs from the ODC overproduction-based resistance to DFMO as there have been no signs indicating that cells selected under the pressure of MGBG start to overproduce AdoMetDC (also our own unpublished results).

4. Combination of Inhibitors of Adenosylmethionine Decarboxylase with Inhibitors of Ornithine Decarboxylase and Conventional Cancer Chemotherapeutic Agents

a. Combination with Inhibitors of Ornithine Decarboxylase

The finding by Alhonen-Hongisto et al.[48] indicating that polyamine depletion, achieved by the inhibition of ODC, led to an enhanced accumulation not only of the natural polyamines but also of MGBG served as the basis for a sequential combination of ODC and AdoMetDC inhibitors, which was ultimately applied also clinically. An exposure of cultured cells to DFMO until putrescine and spermidine depletion leads to a manifold increase in the cellular accumulation of the natural polyamines, unphysiological diamines, and MGBG.[48,163] Under cell culture conditions, polyamine-depleted cells virtually explode when exposed to MGBG.[193] Similarly, a prior treatment with DFMO also strikingly enhances the accumulation of GBG[151] but not of EGBG[169] or more alkylated derivatives of bis(guanylhydrazones).[146]

The sequential combination of DFMO and MGBG was subsequently applied in tumor-bearing animal models. Studies with healthy mice indicated that a prior treatment with DFMO enhanced the accumulation of the subsequently injected MGBG only in selected tissues such as small intestine and bone marrow, whereas in the parenchymal tissues and muscle, DFMO did not influence the accumulation of MGBG.[194] Similarly, upon chronic administration, there was no apparent cumulative accumulation of MGBG with or without DFMO in any of the tissues of healthy mice studied.[195] In mice inoculated with either Ehrlich ascites carcinoma cells[196] or L1210 cells[195] administration of DFMO (in drinking water) prior to MGBG injections seemed to selectively enhance the accumulation of the latter drug in the tumor cells. Using similar experimental settings (mice inoculated with L1210 leukemia cells), Kramer et al.[197] failed to show any selective enhancement of the accumulation of MGBG in the leukemia cells following DFMO pretreatment.

The combination was then tested in a number of experimental tumors. The sequential combination of DFMO and MGBG was found to be therapeutically more effective than the single drugs in L1210 leukemia.[6,23,198] In each case, an apparently synergistic antileukemic effect was achieved with the combination. Similarly, a combination of 6-heptyne-2,5-diamine, another irreversible inhibitor of ODC, with MGBG, gave an additive antitumor effect in L1210 and P388 leukemias.[45]

Experimental prostate cancer appears to be another animal tumor model in which the combination of DFMO and MGBG shows a striking therapeutic synergism. Dunzerdorfer[199] reported that the combination of the two polyamine antimetabolites resulted in a tumor-destructive effect. In their subsequent studies with transplantable prostate cancer Dunzendorfer et al.[200] found that the combination of DFMO and MGBG was not only more effective than MGBG alone, but even less toxic. Interestingly, similar reduction of MGBG toxicity by DFMO was also reported by Herr and Kleiner[201] when using the combination in hormone-resistant prostate adenocarcinoma in rats. Neither DFMO or nontoxic doses of MGBG inhibited tumor growth, yet the combination produced a striking therapeutic effect with 47% early cure rate and total or near total suppression of tumor growth.[201] Similar dramatic destruction of prostatic adenocarcinoma tissue by the combination of DFMO and MGBG was also reported by Dunzendorfer and Knöner.[202]

Another experimental tumor model for which the combined treatment has been applied is mouse renal adenocarcinoma. In this model, the combined DFMO/MGBG not only inhibited tumor growth but reduced metastases and distinctly increased survival.[203] These authors also found that DFMO selectively enhanced MGBG accumulation in the tumor tissue.

Fujimoto et al.[204] applied the combined polyamine antimetabolite regimen to human stomach cancer xenotransplanted into nude mice. Combining DFMO and MGBG at dose levels that were without any antitumor effect as single agents, produced a clear-cut tumor growth suppression, yet after the termination of the treatment there was a rapid regrowth of the tumor.[204] Interestingly, the antitumor effect achieved with the combined DFMO/MGBG regimen was apparently not based on any polyamine depletion, as the polyamine pools remained virtually unaltered during the course of the treatment.[204]

There thus are a number of experimental tumors, in addition to leukemias, which are sensitive to the combined treatment with DFMO and MGBG. What is then the mechanism of the cytotoxic action of the combination? As just mentioned, it is not the polyamine depletion in the xenotransplanted human stomach carcinoma[204] and although the determinations of the tumor polyamines have not been reported in most of the studies cited in this section, it is probable that polyamine depletion is not the reason for the antiproliferative action exerted by the combination. In fact, we have shown[168] that putrescine and spermidine depletion achieved with DFMO in inoculated L1210 cells is totally lost during the treatment with MGBG.

There are quite opposite examples, i.e., experimental tumors which are sensitive to DFMO (or to ODC inhibitors in general) but upon combination with MGBG this sensitivity is lost. In connection with their studies with a murine mammary sarcoma, Prakash et al.[205] observed that when DFMO was combined with MBAG, the irreversible bis(guanylhydrazone) inhibitor of AdoMetDC, putrescine and spermidine concentrations, but not ODC activity, returned to normal values and the antitumor effect of DFMO was lost. In an analogous fashion, heptynediamine (the most potent irreversible inhibitor of ODC) when combined with MGBG showed additive antitumor effect in mouse leukemia but antagonistic effect in mouse mammary sarcoma.[45]

The reasons for this seemingly paradoxal behavior of DFMO-MGBG combination, as regards the polyamine depletion, are experimentally established today. The reversal of DFMO-induced putrescine and spermidine depletion upon administration of MGBG (or other bis(guanylhydrazones) is apparently based on the profound inhibition of DAO (intestinal) activity.[149,156,158,159] The consequences of the inhibition of intestinal DAO activity is schematically presented in Figure 4. The high DAO activity of the intestine is virtually abolished by therapeutic doses of MGBG[158] leading the bacteria-derived diamines and polyamines to

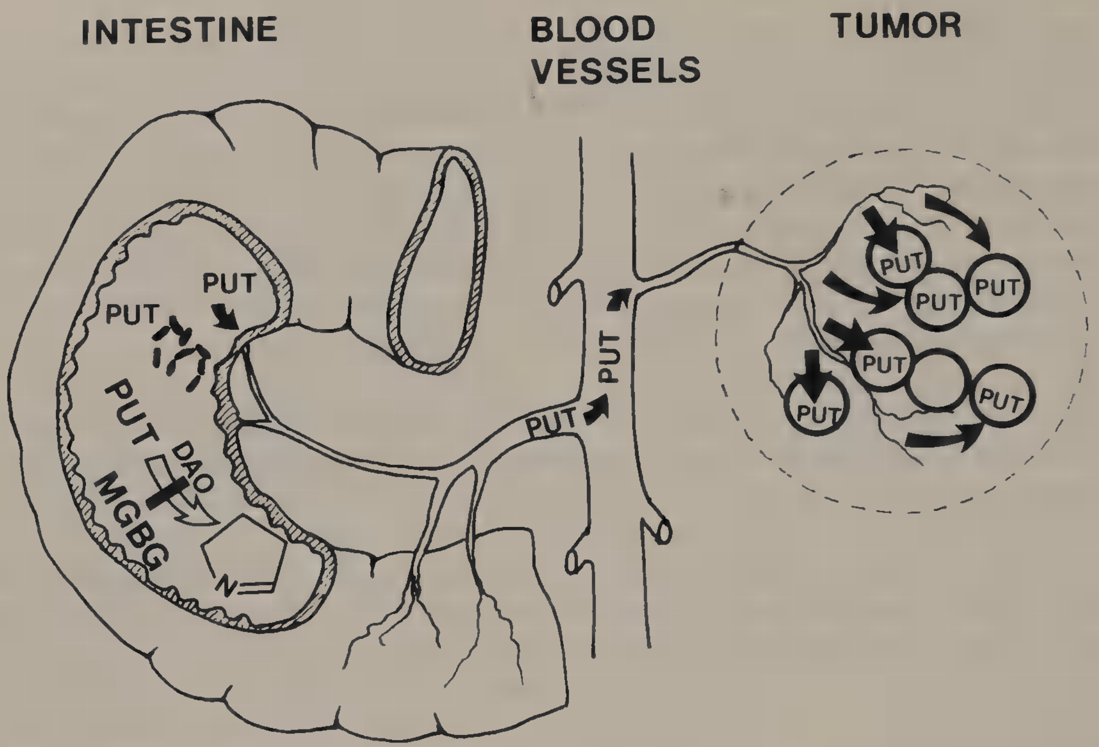

FIGURE 4. Schematic representation of the effect MGBG-induced inhibition of intestinal DAO combined with DFMO-induced enhanced uptake of polyamines on the reversal of polyamine depletion in the tumor cells.

enter systemic circulation and finally into the polyamine-depleted tumor cells exhibiting strikingly enhanced capacity to accumulate extracellular polyamines.[159] Thus, in case of tumors sensitive to DFMO but resistant to MGBG (as many of the solid tumors are) the DFMO-induced growth-inhibition is lost. However, tumors sensitive to MGBG (leukemias and lymphomas) can be effectively treated with the combination, yet DFMO is only used as a vector to enhance the accumulation of MGBG the cytotoxicity of which (having nothing to do with the production of polyamines) is finally responsible for the antitumor effect.

Apart from MGBG, the other bis(guanylhydrazones) have not been used in combined regimens with DFMO in experimental cancer models. There is, however, one interesting exception, Shrestha et al.[206] compared the efficacy of DFMO-MGBG combination with DFMO-EGBG combination (EGBG is classified as being devoid of antileukemic activity) in xenografts of human stomach cancer and found that the latter combination was more effective than DFMO-MGBG, especially as regards the tumor regrowth after cessation of the treatment. This preliminary finding requires confirmation and more extensive documentation.

b. Combination with Conventional Cancer Chemotherapeutic Agents

Few studies have been directed to the combined use of bis(guanylhydrazones) with conventional cancer chemotherapeutic agents. Fujimoto et al.[204] added mitomycin C on the top of DFMO-MGBG regimens and were able to prevent the tumor regrowth by this combination. In connection with their studies on the possible radiosensitation by polyamine depletion Seidenfeld et al.[97] also tested MGBG in this regard. The compound, however, did not enhance the cell kill after exposure to X-rays.

c. Polyamine Antimetabolite Combination in Hyperproliferative Skin

In addition to various neoplasms, polyamine antimetabolites may have potential in the treatment of hyperproliferative skin diseases, most notably of psoriasis. After the development of the DFMO priming concept, the combination of DFMO and MGBG was also tested in mouse skin induced to proliferate by an exposure to UV light. As in case of cultured tumor cells, a prior treatment with topical DFMO greatly enhanced the epidermal accumulation of topically applied MGBG in mouse skin.[207-209] In case of extensive irritation by MGBG, we also developed a rescue concept in which high epidermal MGBG concentrations could be rapidly reduced by topical spermidine.[209]

Although the combination of DFMO and MGBG is effective in depressing the proliferative activity of mouse skin, this combination has apparently not been applied clinically.

5. *Clinical Studies with Bis(Guanylhydrazones)*

There are some interesting features in the clinical trial program currently going on with MGBG. All the experimental data produced so far indicate that MGBG displays strong antitumor activity against rapidly proliferating tumor cells but is virtually ineffective against slower growing solid tumors (see Section II.B.3). The accumulation of cytotoxic drug levels is likewise critically dependent on the growth rate of the exposed cells.[165] Unlike in rodents,[141] the drug in man shows cumulative toxicity.[6] However, if the 24-hour urinary excretion of MGBG in adults does not exceed 16%[210] this may not be the case in children whose urinary excretion appears to be substantially higher.[164] Furthermore, the very first clinical trials indicated that MGBG was strikingly effective in acute leukemia.[211-213] These early clinical trials also revealed that MGBG possesses an extremely narrow therapeutic index, yet being possibly better tolerated by children than by adults.[213] Simple methods are available to determine the drug levels in peripheral leukocytes (the plasma clearance of MGBG is so rapid that determination of plasma levels is of no use),[164,185,214] which can be used to predict the toxicity.[165] Now comes the 1000-dollar question! The current reevaluation program of MGBG almost exclusively involves adult patients with solid tumors, no leukemias, a few lymphomas, a few children, and, needless to say, nobody seems to be interested in determining the actual cellular drug levels to predict toxicity.

As the early clinical studies with MGBG as single agent has been recently reviewed by Warrell and Burchenal[6] and by Jänne et al.[5] only a brief summary is given here. In the early clinical studies the drug was administered daily. This treatment schedule displayed striking activity in acute leukemia producing complete remission up to 69%, yet at the cost of severe toxicity.[5,6] Out of more than 100 patients with acute myeloblastic, or lymphoblastic, or chronic myelocytic leukemia more than 25% responded to MGBG treatment.[6] The response rate of patients with malignant lymphoma was even better, i.e., more than half of the patients responded to single-agent MGBG.[6] Among the solid tumors, the response rate was less than 15%.[6]

The reevaluation of the drug started in 1979 when Knight et al.[215] published their trial with MGBG now using, instead of daily dosing, a weekly administration schedule. Until now probably more than 1000 cancer patients have received MGBG as a single-agent treatment with intermittent administration schedule. The vast majority of patients has had solid tumors with low response rate. Again, best responses were achieved in patients with malignant lymphoma.[6]

Since the completion of the two review articles[5,6] the interest in MGBG, if anything, seems to be fading somewhat. The latest clinical trials (phase II) indicated that single-agent MGBG had no or only marginal activity in nonsmall cell carcinoma of the lung,[216] relapsed small cell carcinoma,[217] advanced ovarian cancer,[218] refractory germ cell tumors,[219] or soft-tissue sarcomas.[220] Very recently, the U.S. pediatric oncology group[221] reported a phase II trial of MGBG in children with relapsed leukemia and lymphoma. The fact that MGBG did not show any therapeutic efficacy is attributable, in addition to the possibility that the drug is not effective at all, to the rather low dose used, as in fact discussed by the authors.[221] As already mentioned, children appear to tolerate substantially higher doses of the drug than the adults do.

In contrast to the earlier conclusions,[6] Thongprasert et al.[222] found MGBG only marginally active in patients with advanced head and neck cancer.

In spite of these negative therapeutic results, a limited number of human cancer types seems to respond to MGBG at an acceptable toxicity level. The earlier observations indicating that MGBG exhibits significant antitumor activity in heavily pretreated lymphoma patients have been repeatedly confirmed.[223] In agreement with studies in animal models, weekly MGBG was likewise found to induce partial remissions in 24% of patients with hormone-resistant prostatic carcinoma.[224]

In addition to our pharmacokinetic trial with the sequential combination of DFMO and MGBG in childhood leukemia,[124,165] only a few trials have been carried out with the combination. Warrell et al.[225] employed the sequential combination in patients with lymphomas and solid tumors, and reported substantially more toxicity than experienced restrospectively with MGBG alone with minimal therapeutic activity. Similarly Splinter and Romijn[226] found the combination toxic in adult patients with various, mainly solid, tumors, with marginal therapeutic efficacy. However, some preliminary observations seem to indicate that the combination of DFMO and MGBG may be effective in the treatment of brain tumors, yet these reports are still at the anecdotal level.[227,228]

During the last few years, MGBG has also been included in various multidrug regimens with conventional cancer chemotherapeutic agents. Even though it understandably is extremely difficult to evaluate the therapeutic contribution of MGBG in these regimens, it appears that good therapeutic responses can be achieved in esophageal cancer.[229,230] Incidentally, MGBG as a single agent has shown substantial therepeutic activity in this particular cancer.[6] A combination of MGBG with cisplatin and amsacrine was tested as salvage treatment of advanced non-Hodgkin's lymphoma. Although objective responses were recorded in almost half of the patients, these were of short duration and apparently obtained at the cost of severe toxicity.[231]

What are the conclusions regarding the clinical usefulness of MGBG as a single agent or as a component of various multidrug regimens? One thing is certain, MGBG with its narrow therapeutic index is and also remains as a tricky drug, the trickiness of which has not been properly appreciated by the clinicians. Moreover, probably the whole clinical reevaluation program during the past few years has taken a wrong direction. Based on these trials as well as on experimental studies, it appears obvious that it is no more justifiable to treat a large number of patients with a random variety of solid tumors with MGBG as this only adds more details to the toxicological picture of the drug. Admittedly, there are a few solid tumors, such as esophageal and prostatic cancer, in which the therapeutic potential of MGBG should be carefully investigated to confirm the earlier studies. Thus the lymphomas and probably also leukemias must be the logic target neoplasms for further clinical studies.

There apparently are also pharmacokinetic means to predict the toxicity of MGBG. Pharmacokinetic studies in man as well as the accumulated clinical experience unambiguously have shown that this drug, due to its slow excretion rate,[232,233] causes cumulative toxicity in man. Most of MGBG is cleared from the plasma relatively rapidly,[164,232,233] thus making the determinations of plasma drug concentrations virtually useless as a means to predict proper therapeutic dose level and toxicity. However, the drug appears to be effectively retained by peripheral mononuclear leukocytes.[124,164] Accordingly, the determination of MGBG concentrations in leukocytes, which can be done indirectly without actually isolating the ''buffy-coat'' cells[185,214] would probably give an idea of the tissue levels of the drug and thus help to adjust the dose and avoid cumulative toxicity.

In conclusion, although MGBG will never gain the position of a first-line cancer chemotherapeutic agent, it is an indispensable model compound with its unique mode of action.

C. Inhibitors of Spermidine and Spermine Synthases

In contrast to the mouting number of experimental and even clinical studies dealing with the inhibitors of ODC and AdoMetDC, much less attention has been paid to the development of inhibitors of the propylamine transferases. The reasons for this are obvious. The two decarboxylases are clearly rate-controlling enzymes as regards the formation of spermidine and spermine. Moreover, growth inhibition, at least cytostasis, can be achieved through a depletion of putrescine and spermidine only with inhibitors of ODC in spite of unchanged or increased levels of spermine.[4] Thus the development of inhibitors of spermine synthase mainly serves as a means to elucidate the role of spermidine in cellular proliferation. Furthermore, a suppression of spermidine formation through a specific inhibition spermidine synthease seems to be unnecessary as the formation of this polyamine can be blocked by inhibitors of ODC. Apparently, none of the inhibitors of the propylamine transferases has been tested in experimental tumor models.

Probably the oldest inhibitor of propylamine synthases was 5′-methylthioadenosine (MTA) which inhibits spermine synthease from various mammalian sources.[234,235] However, although MTA and its alkylated derivatives decreased the concentration of spermine and inhibited cell growth, the antiproliferative effects were not reversed upon an addition of exogenous polyamines.[236] Wang et al.[237] synthesized an AdoMet derivative, 2-methyl AdoMet. Although reported as an inhibitor of AdoMetDC, this compound selectively blocked the formation of spermine in activated lymphocytes with concomitant depression of DNA synthesis.[237] Pegg and Coward[238] recently showed that a derivative of MTA, namely *S*-methyl-MTA, blocked the synthesis of spermine in SV-3T3 cells. Interestingly, this compound, although producing a profound spermine depletion (with some increase in spermidine content), did not influence the growth of the cells.[238] Together with other pieces of evidence, this phenomenon, i.e., the lack of growth inhibition, again suggests that spermine may not be needed for mammalian cell proliferation to occur.

Out of the inhibitors of spermidine synthase, dicyclohexylamine[239] and the transition-state analogue *S*-adenosyl-3-thio-1,8-diaminooctane (AdoDATO[240]) are best characterized. Both compounds effectively block the formation of spermidine associated with a substantial rise in putrescine and spermine contents.[241-243] When combined with DFMO, AdoDATO effectively lowered spermine content resulting in a depletion involving all three polyamines.[241] However, the almost total growth inhibition produced by DFMO alone was not potentiated by AdoDATO.[241]

Pegg et al.[244] tested a series of 5′-substituted adenosines for a possible inhibition of the two propylamine transferases. Although some of the compounds were shown to be potent and selective inhbiitors of spermine formation, the latter authors[244] reached the conclusion that inhibitors of the two propylamine transferases (AdoDATO and these new inhibitors of spermine synthase) are useful as antiproliferative agents only under conditions where the compensatory increases of decarboxylated AdoMet levels can be blocked by effective inhibitors of AdoMetDC.

In summary, the two propylamine transferases (spermidine and spermine synthases) are apparently not meaningful targets for an effective cancer chemotherapy, at least without the availability of effective and specific inhibitors of AdoMetDC and ODC. Regarding the role of spermine in cell proliferation, much of the experimental evidence suggests that this compound may not be required for growth but merely represents a sort of salvage compound, for which a machinery exists to effectively convert it back to spermidine and putrescine. The experimental evidence suggesting that spermine is not necessary for cell proliferation to occur, include, among others, the following facts:

1. Spermine is not essential for life as many of the bacteria and lower eukaryotes do not contain spermine.
2. Depletion of putrescine and spermidine is sufficient to cease growth even under conditions where spermine levels are higher than normal.

3. Selective inhibition of spermine formation does not lead to growth inhibition.[238,244] Yet, one must admit that severe spermine depletion is characterized by compensatory increases in the contents of putrescine and/or spermidine.[238,244]
4. Finally, the almost historical observation, which has been repeatedly confirmed during the past 20 years, indication that a high ratio of spermidine to spermine is indicative of a rapid proliferation rate[245] also emphasizes that spermidine, but possibly not spermine, is important for growth.

III. CONCLUDING REMARKS: IS THERE A ROLE FOR POLYAMINES IN CANCER CHEMOTHERAPY?

The use of a prevention of polyamine accumulation as an approach to the chemotherapy of hyperproliferative diseases, most notably of cancer, has developed in a different way in comparison with most drugs currently used clinically. It is more a rule than an exception that most of the conventional cancer chemotherapeutic agents have been discovered among exotic compounds on the basis of their antiproliferative effects in various anticancer screens. The mode of action and the possible target reactions have been of secondary importance and have been elucidated only a number of years later. This approach largely represents a random screen as the derivatives of the given active compound have been designed without the important information of the mode of action of the drug. In the case of inhibitors of polyamine biosynthesis, the experimental basis was established years before the first polyamine antimetabolite was to be discovered. However, the initial enthusiasm and excitement followed by the experiments indicating that polyamine depletion, achieved by seemingly simple chemical intervention of their biosynthetic enzymes, was, or at least should have been, tempered by repeated observations showing that it is not a single reaction, but a system, one has to fight against. The system in this connection refers to the bewildering sophistication of the compensatory reactions triggered by a depletion of one or two of the natural polyamines. In addition to secondary inductions of other biosynthetic enzymes in response to the inhibition of one of them and to gene amplification based overproduction of the target enzyme (ODC), the compensatory reactions also include changes in cellular accumulation and excretion of the polyamines and even changes involving enzymes not really directly related to the biosynthesis of the polyamines. Thus the role of diamine oxidase has become a very central one, when AdoMetDC inhibitors are used. Moreover, the role of arginase is gaining. Our unpublished experiments indicate that in the absence of ODC overproduction the resistance to DFMO can be developed on the basis of a striking increase in the activity (and production?) of arginase providing more ornithine to compete with DFMO.

Is there or will there be room for inhibitors of polyamines biosynthesis among the cancer chemotherapeutic agents? The answer is affirmative. Even at the current level of knowledge, it is more than clear that the clinical potential of the existing polyamine antimetabolites, most notably DFMO and other inhibitors of ODC or MGBG and its congeners has not been explored in depth. It is likewise obvious that the combination of the inhibitors of polyamine biosynthesis with more conventional cancer chemotherapeutic agents has not really started yet, as the information, also largely including experimental studies, is rather fragmentary with no apparent intention to real dose-dependence studies. ,

We are confident that the inhibitors of polyamine biosynthesis will find their place, probably a central place, among the modern cancer chemotherapeutic agents. However, we also believe that abbreviations such as DFMO or MGBG in modern cancer chemotherapeutic regimens will be replaced by other acronyms that are not known at the moment of writing.

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Chapter 16

CLINICAL STUDIES OF α-DIFLUOROMETHYLORNITHINE AND α-INTERFERON COMBINATION IN CANCER PATIENTS

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TABLE OF CONTENTS

I. Introduction 288

II. Results 288

III. Discussion 290

Acknowledgments 291

References 291

I. INTRODUCTION

α-Difluoromethylornithine (DFMO) is an irreversible inhibitor of ornithine decarboxylase (ODC), the rate-limiting enzyme in the polyamine biosynthetic pathway which converts ornithine to putrescine. Polyamines were demonstrated to be essential for normal cellular growth and malignant cell growth as well.[2-4] Subsequently, DFMO was shown to exert antiproliferative activities on several tumor cell lines and animal tumor models.[5-7]

α-interferons (IFNs) are naturally occuring proteins with pleiotrophic activities, such as antiviral, immune-stimulating, and antiproliferative actions.[8-12] Its antitumor activities were studied extensively and were demonstrated both in vitro and in vivo.[13-16]

A growing body of evidence alluded to enhanced antitumor activity of the combination of DFMO and IFN-α when compared to either DFMO or IFN-α alone.[17-19] We have decided, therefore, to examine the clinical activity of the combination of DFMO and IFN-α.

In this chapter, we will summarize the clinical studies conducted with the combination at M.D. Anderson Hospital and Tumor Institute.

II. RESULTS

In the first phase I study,[20] we have examined the toxicity, tolerance, and activity of escalating doses of DFMO administered orally combined with i.m. partially pure IFN-α (Finnish Red Cross, Helsinki, Finland) (Table 1). Increasing toxicities were demonstrated with the increased dose of DFMO and IFN-α. Gastrointestinal toxicites, including diarrhea, nausea, and occasional vomiting, consisted of the most common and disturbing toxicites. The maximal tolerated dose (MTD) of the combination was found to be 3.2×10^6 U/m^2 of IFN-α daily and 6 g/m^2 of DFMO daily. The individual contribution of oral DFMO to the gastrointestinal toxicities was later demonstrated when seven additional patients were given lower than the MTD doses of IFN-α (1.6×10^6 U/m^2) but higher doses of DFMO (8 g/m^2 daily). Nearly 50% of these patients developed unacceptable gastrointestinal toxicities.

Less frequent toxicities included thrombocytopenia with platelet counts <100,000/mℓ which developed in five patients and required 3 to 5 days of treatment interruption for recovery. Anemia with hemoglobin levels of <10 g/dcℓ developed in three patients.

A unique toxicity was manifested by the development of neurogenic hearing loss in 18 of 25 patients after a median period of 6.4 weeks (range, 2 to 10 weeks). The median total dose of DFMO given prior to the development of hearing loss was 587 g (range, 142 to 1101 g). This toxicity was, however, reversible and discontinuation of treatment led to hearing improvement after a median period of 8.7 weeks (range, 3 to 12 weeks).

The clinical activity of the combination is demonstrated in Table 2. Of the patients with metastatic melanoma (12 patients), one patient achieved complete remission of a metastatic tumor in the lungs and mediastinum and has had no evidence of recurrence for 30+ months since the initiation of treatment. A second patient with brain, liver, and skin metastatic disease was defined as having partial remission because biopsy of a skin nodule during therapy continued to demonstrate presence of melanoma. However, this patient, also, has had no evidence of disease recurrence off therapy after 30+ months since the initiation of treatment.

In order to examine whether i.v. DFMO administration will reduce the gastrointestinal side effects, we have examined the tolerance of continuously infused i.v. DFMO combined with i.m. IFN-α. Three doses of DFMO were examined: 6, 8, and 10 g/m^2/day. A single IFN-α dose of 3×10^6 units daily was given to all patients. Thrombocytopenia of 100,000/mℓ developed in all 19 studied patients after a median period of 17, 20, and 18 days (for

Table 1
TREATMENT DOSES AND CLINICAL SIDE EFFECTS OF ORAL DFMO AND IFN-α

Dose levels					
IFN-α $\times 10^6$u/m^2	**DFMO g/m^2/day**	**GI symptoms (grades 2 and 3)**	**Fatigue (grades 2 and 3)**	**Anemia <10 g%**	**Thrombocytopenia ($<100 \times 10^3/\mu\ell$)**
0.4	6	1/6	1/6	0/6	1/6
0.8	6	2/8	2/8	0/8	0/8
1.6	6	2/11	4/11	1/11	0/11
3.2	6	3/14	4/14	2/14	0/14
6.4	9	10/22	5/22	1/22	4/22
Total		18/61	16/61	3/61	5/61

Table 2
CLINICAL RESPONSES ON DFMO AND IFN-α

Disease	Patients studied	Complete remission	Partial remission	Minor response	Progressive disease
Malignant melanoma	12	1	1	3	7
Renal cell carcinoma	4	0	0	1	3
Colon cancer	4	0	0	1	3
Chronic myeloagenous leukemia	3	0	0	3	0
Others	2	0	0	0	2
Total	25	0	2	8	15

Table 3
THE DEVELOPMENT OF THROMBOCYTOPENIA ON THREE DIFFERENT DOSE SCHEDULES OF INTRAVENOUS DFMO AND α INTERFERON

Daily DFMO dose (g/m)	Number of courses	Median (range) number of days to thrombocytopenia	Platelet ($\times 10\alpha/\mu\ell$) nadir	Median number of days to recovery from thrombocytopenia
6	9	17 (11—21)	32 (13—63)	8 (7—13)
8	7	20 (15—47)	52 (32—89)	8 (7—12)
10	3	18 (16—20)	70 (43—78)	13 (6—13)

6, 8, and 10 g of DFMO, respectively) (Table 3). The time to recovery ranged from 8 to 13 days. The thrombocytopenia associated with repeated courses of DFMO was examined in patients who received more than one treatment course (Table 3). Thus, comparison between the first and second courses of treatment demonstrates a slight decrease in the cumulative dose of DFMO that could be administered prior to the development of thrombocytopenia, and also a slight reduction in the treatment duration before the development of thrombocytopenia during the second course of treatment.

Hearing loss developed in three of the nine patients studied. The median dose of DFMO to the development of this toxicity was 791 g (764 to 820 g). Gastrointestinal toxicities such as diarrhea, nausea, and weight loss which were observed with the oral administration of DFMO have not been seen with i.v. administration of DFMO.

In a recent study, we decided to alternate between the doses of DFMO at 6 and 4 g/m^2 in an effort of prevent treatment interruption due to thrombocytopenia. Preliminary data suggest that some patients can be treated continuously for 2+ months on this regimen.

Table 4

SUPPRESSION OF RED CELL POLYAMINES IN CANCER PATIENTS TREATED WITH α INTERFERON, α INTERFERON COMBINED WITH ORAL DFMO, AND α INTERFERON COMBINED WITH INTRAVENOUS DFMO

	No. of patients studied	Suppression putrescine (median time to max. suppression)	Suppression of spermidine (%)	Suppression of spermine (%)
IFN-α	36	42.8 ± 18.5	40.2 ± 23.1	53.6 ± 19.3
IFN-α + DFMO (oral)	14	66.5 ± 22.4 (7 days)	36.7 ± 30.2 (9 days)	30.9 ± 24.4 (14 days)
IFN-α + DFMO (i.v.)	11	72 ± 14 (22 days)	67 ± 12 (20 days)	43 ± 20 (16 days)

Preliminary data on clinical responses with i.v. DFMO combined with IFN-α show activity in metastatic melanoma among the 13 patients studied. A partial remission was demonstrated in two patients with soft tissue metastases (lasting 6+ months).

Red blood cell putrescine, spermidine, and spermine levels were determined on a Durrum-Dionex D-500 amino acid analyzer (Dionex Corp, Palo Alto, Calif.) by methods described previously.[21] Recovery of putrescine, spermidine, and spermine was 85, 78.4, and 86.5%, respectively, and values were corrected for these percentages of recovery.[21] The degree of red cell polyamine suppression was examined in patients receiving IFN-α, IFN-α combined with oral DFMO, and IFN-α combined with i.v. DFMO. These studies (Table 4) demonstrated an increase in the inhibition of putrescine following the addition of oral DFMO to IFN-α. Treatment with i.v. DFMO markedly increased the inhibition of putrescine and spermidine over that of oral DFMO and IFN-α. Interestingly, the time to development of thrombocytopenia (on i.v. DFMO and IFN-α) (between 17 to 20 days) overlaps with the time required for maximal polyamine suppression (between days 16 and 22).

III. DISCUSSION

In a series of phase I-II clinical studies, we examined the toxicities and clinical activities among the patients treated with the combination of DFMO and IFN-α. Marked gastrointestinal toxicity was observed with the administration of oral DFMO presumably due to direct effect of the DFMO on the gut mucosa, thereby, leading to severe diarrhea and occasional vomiting. The alteration of the route of DFMO administration from oral to i.v. led to complete abolishment of the gastrointestinal toxicities. With the use of i.v. DFMO, we have noticed reproducible thrombocytopenia which occurred infrequently with the oral DFMO. We feel that these events indicate general polyamine suppression which was not observed frequently with oral DFMO, perhaps because of low levels of DFMO in the circulation.

The time to thrombocytopenia correlated with the time to maximal red cell polyamine suppression, thus perhaps serving as a biologic marker for adequate continuous suppression of total body polyamines. Besides the thrombocytopenia, i.v. administration of DFMO was essentially devoid of side effects and patient tolerance to this approach was excellent. We concluded that i.v. administration of DFMO is superior to oral administration and thrombocytopenia can be prevented by either DFMO dose alternations, or treatment interruption.

Neurogenic hearing loss was a frequent toxicity of oral DFMO administration, but was reversible following treatment interruption. This toxicity appears to correlate with cumulative dose of DFMO.

In these preliminary studies, we have demonstrated clinical activity against metastatic melanoma, the tumor that was most extensively studied. However, phase II studies to examine the activity of DFMO + IFN-α in melanoma are clearly required to confirm the preliminary observations. Also, malanoma appears to be a suitable solid tumor model to examine whether the combination of DFMO and IFN-α is superior to IFN-α alone. The latter, in particular, the recombinant α-interferons have low but definite antitumor activity in melanoma.[22,23] Thus, future Phase II studies can compare the activity of the combination to recombinant α-A interferon alone.

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Chapter 17

CHANGES IN POLYAMINE METABOLISM IN TUMOR-BEARING HOSTS WITH TOTAL PARENTERAL NUTRITION AND INTRAVENOUS INFUSION OF α-DIFLUOROMETHYLORNITHINE

Kenji Nishioka, V. Bruce Grossie, Jr., David M. Ota, and Jaffer A. Ajani

TABLE OF CONTENTS

I. Introduction 294

II. Materials and Methods 294

III. Results 295

IV. Discussion 297

Acknowledgments 297

References 299

I. INTRODUCTION

Malnutrition and cachexia are negative prognostic factors for the survival of cancer patients.[1-3] Total parenteral nutrition (TPN) is an effective method of feeding those patients who are unable to take food orally. Despite correction of nutritional deficits with TPN, however, improved host survival has not been observed.[4,5] This result may be related to the competition of normal host tissues and malignant cells for nutrients. While stimulation of tumor growth by TPN has never been documented in humans, the results obtained from animal experiments have been inconsistent.[6-8] One problem in the extrapolation of data from animal studies to cancer patients is the lack of an indicator which predicts tumor metabolism and which can be measured in both patients and animals. In view of this, we have used erythrocyte (RBC) polyamine levels as markers for tumor growth.[9] In our patient study, we observed that TPN was associated with significant increase in RBC putrescine levels in cancer patients but not in normal controls, suggesting possible TPN-induced tumor proliferation. In our recent study, we have identified several patients who showed a clear increase of RBC putrescine upon TPN treatment. They all had a history of ''curative'' operation and were subsequently shown to have recurrent disease.

In order to study the possible relationship between RBC polyamine levels and tumor growth, we have resorted to an animal model in which tumor-bearing rats are malnourished by restricted intake (RI). First, in this model we examined the effect of TPN on tumor growth and polyamine metabolism in the hosts. Second, we attempted to determine if the simultaneous administration of TPN with α-difluoromethylornithine (DFMO) can improve host utilization of nutrients while inhibiting tumor growth, taking advantage of possible differential requirements of polyamines between normal and tumor cells.

II. MATERIALS AND METHODS

A transplantable methylcholanthrene-induced fibrosarcoma (0.17 g) was inoculated subcutaneously into the right flank of male Fisher 344 rats (Harlan-Sprague Dawley, Inc.) under anesthesia. After the tumors were at least 1.0 cm in width, two-dimensional measurements were taken three times weekly and tumor weights calculated.[10,11] Restricted intake of chow (8 g/day) was fed daily.

To initiate a continuous infusion, rats were randomized by tumor weight, and a central venous catheter inserted under general anesthesia as previously described.[11] The animals were allowed to recover overnight and infusions were started the next morning, using a Holter 903 infusion pump (Critikon) to administer solutions at a constant rate (40 to 50 mℓ/day). DFMO (Merrell-Dow Research Institute) was constituted in 0.45% NaCl, or added directly to TPN solutions. The TPN regimen consisted of Travasol 10% (Travenol Laboratories Inc., 500 mℓ), 60% dextrose (Abbott Laboratories, 500 mℓ), TPN electrolytes (Abbott, 20 mℓ), potassium phosphate (6 mℓ), and multiple vitamins (2 mℓ).

After a continuous infusion, all rats were anesthesized with Ketamine, exsanguinated through the abdominal aorta into a vacutainer tube with heparin, and the tubes placed on ice. The tumor and liver were removed and weighed. The liver and necrosis-free tumor tissue were frozen at −70°C for future analysis of ornithine decarboxylase (ODC) activity and polyamine levels. The blood was centrifuged and the plasma stored for albumin determination. RBC and tumor were extracted for polyamine analysis as previously described.[11,12] The ODC activity of liver and tumor was analyzed as described by Harris et al.[11] Plasma albumin levels were determined spectrophotometrically using the bromcresol-green-binding assay (American Monitor Corp.).

All data are reported as the mean ± SD. Statistical evaluation of the differences between means was performed by the Student's *t* test.

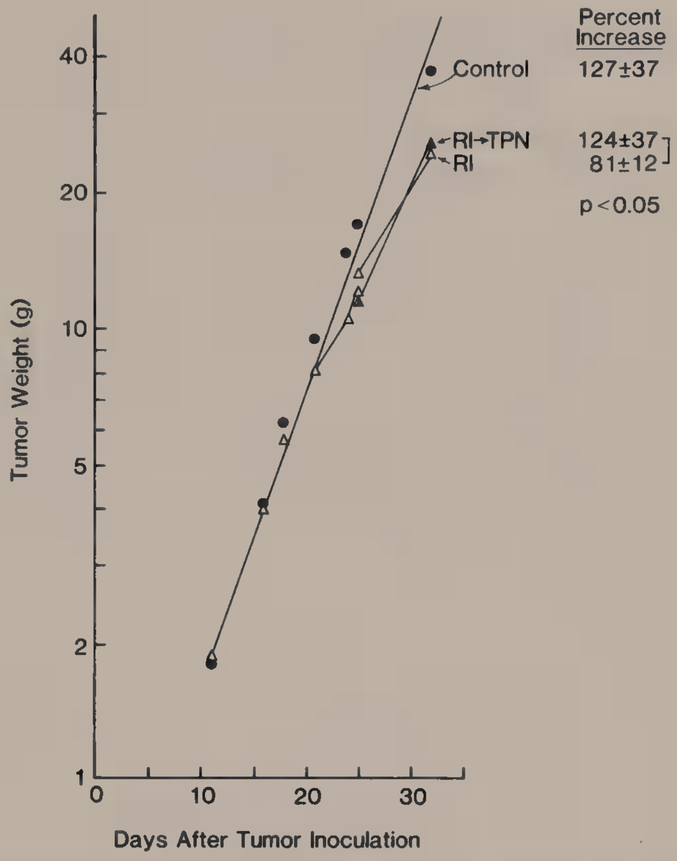

FIGURE 1. Growth of a fibrosarcoma in rats fed chow ad libitum (control: ●), a restricted intake of chow (RI: △) for 21 days, or a restricted intake of chow for 14 days followed by 6 days of TPN (RI → TPN: ▲).

III. RESULTS

As shown in Figure 1, tumors of control rats which were fed chow ad libitum, grew exponentially throughout the study, whereas the tumor growth rate of RI rats decreased during the initial 14 days with the final tumor weights being significantly lower than those of the controls. When the malnourished rats were given TPN for 6 days after 14 days of RI regimen, tumor weight increased significantly compared with those rats fed the RI regimen for the entire 21 days. The tumor ODC activity of RI rats was significantly lower compared with the controls (Table 1). The effect of induced malnutrition on tumor putrescine levels paralleled the tumor ODC activities. Tumor spermidine and spermine levels were not affected. TPN repletion resulted in consistant increases in tumor ODC and putrescine levels compared with the tumors of rats continued on the RI regimen. The effect of the malnutrition on the RBC polyamines was then examined (Table 2). The RBC polyamine levels were significantly reduced when the RI regimen was employed for 21 days. TPN resulted in a consistant increase in all RBC polyamines. In this study hepatic polyamine metabolism was also studied. Induced malnutrition resulted in a significant reduction in hepatic ODC (17.4 ± 1.4 to 10.9 ± 1.7 in nmol/hr/g wet tissue). The 6-day TPN treatment, however, failed to restore the ODC activity. On the other hand, TPN increased plasma albumin levels, a potential indicator of nutritional status, significantly.

In a separate series of experiments, we have examined antitumor effect of DFMO given by continuous i.v. infusion employing this animal model. Inhibition of tumor growth was time and dose dependent. Although some inhibition of tumor growth was observed in all schedules employed, 12 days of the continuous DFMO infusion particularly exhibited significant suppression of tumor growth (Table 3). The higher doses of 12-day DFMO infusion

Table 1
EFFECT OF INDUCED MALNUTRITION AND TPN ON TUMOR ODC AND TUMOR PUTRESCINE LEVEL

Regimen	No.	Tumor ODC[a]	Tumor putrescine[b]
Control	5	4.2 ± 0.9	39.8 ± 12.0
RI	6	1.9 ± 0.4[c]	30.4 ± 5.7
RI → TPN	7	8.1 ± 2.1[d]	56.5 ± 4.1[d]

[a] nmol CO_2/hr/g wet tissue, mean ± SD.
[b] nmol/g wet tissue, mean ± SD.
[c] Means differ significantly as compared with control rats.
[d] Means differ significantly as compared with RI rats.

Table 2
EFFECT OF HOST NUTRITIONAL STATUS ON RBC POLYAMINE LEVELS

Regimen	No.	Putrescine[a]	Spermidine[a]	Spermine[a]
Control	5	1.4 ± 0.2	46 ± 23	4.4 ± 0.7
RI	6	1.0 ± 0.2[b]	20 ± 6[b]	2.9 ± 0.4[b]
RI → TPN	7	2.9 ± 0.4[c]	175 ± 68[c]	13.2 ± 6.4[c]

[a] nmol/mℓ packed RBC, mean ± SD.
[b] Means differ significantly as compared with control rats.
[c] Means differ significantly as compared with RI rats.

Table 3
EFFECT OF A CONTINUOUS I.V. INFUSION OF DFMO ON TUMOR GROWTH, PLATELET COUNTS, AND PUTRESCINE LEVELS IN TUMOR AND RBC

Dose	Infusion days	No.	% Increase in tumor weight[b]	Platelet counts[c]	Tumor putrescine[d]	RBC putrescine[e]
0[a]	6	4	133 ± 29	881 ± 484	35.9 ± 14.9	0.72 ± 0.10
400 ± 37		5	105 ± 32	661 ± 105	1.8 ± 0.7[f]	0.15 ± 0.02[f]
837 ± 155		3	78 ± 24[f]	508 ± 108	2.1 ± 0.5[f]	0.14 ± 0.01[f]
1696 ± 232		5	94 ± 21[f]	390 ± 77[f]	0.9 ± 0.4[f]	0.09 ± 0.01[f]
0[a]	12	5	371 ± 66	745 ± 96	33.0 ± 8.8	1.00 ± 0.19
425 ± 61		3	204 ± 26[f]	958 ± 486	2.1 ± 0.7[f]	0.24 ± 0.08[f]
916 ± 59		4	142 ± 55[f]	349 ± 29[f]	1.2 ± 0.4[f]	0.16 ± 0.03[f]
1697 ± 190		5	104 ± 46[f]	61 ± 55[f]	1.4 ± 0.5[f]	0.18 ± 0.04[f]

[a] 0.45% NaCl was administered to control rats at the same rate used for the DFMO infusions.
[b] Percent increase in tumor weight during DFMO treatment.
[c] $\times 10^3/mm^3$.
[d] nmol/g wet tissue.
[e] nmol/mℓ packed RBC.
[f] Means differ significantly as compared with 0.45% NaCl-infused rats.

also showed significant decreases in platelet counts. At the nontoxic dose of 425 mg/kg/day for 12 days, a 94% reduction of tumor putrescine levels was achieved with 76% reduction in RBC putrescine.

Based on the above experiments, we have carried out a preliminary experiment in which TPN and DFMO were administered simultaneously. In this preliminary trial, rats were fed a chow diet for 21 days after inoculation of the tumor. Thereafter, TPN and DFMO (1 g/kg/day for 6 days) were administered simultaneously. As shown in Table 4, administration of TPN and DFMO resulted in lower tumor weight compared with TPN alone. Plasma albumin levels and hepatic ODC activity were higher in the TPN + DFMO treated group compared with TPN alone. Tumor ODC activity, tumor putrescine, and RBC putrescine levels were all significantly lower in the TPN + DFMO group compared with TPN alone. Thus, the data suggest that DFMO administration during TPN can selectively inhibit tumor polyamine biosynthesis and may improve host utilizaton of nutrients.

IV. DISCUSSION

As mentioned in Section I, this series of experiments has been performed to examine two major aspects: the effect of TPN on tumor growth and possible modification of this effect with a polyamine antimetabolite, DFMO. Our experimental results suggest a close association of an increase in RBC putrescine levels with an increased tumor growth upon administration of TPN to the malnourished tumor-bearing rats. This observation may relate to possible increase in tumor cell proliferation in patients given aggressive TPN treatment for cancer-related cachexia.[9] In order to minimize this negative effect of TPN, an increase of tumor cell proliferation during the treatment, we have brought DFMO into this system. Our preliminary results indicate that the presence of DFMO in TPN solution somewhat slows down the tumor growth. To ascertain this observation, we are currently examining this cytostatic effect of DFMO employing rats with RI diet and longer infusion period. Our results have also demonstrated that RBC polyamines may be good markers reflecting the status of tumor during TPN, DFMO, or TPN + DFMO treatment. In addition, our results indicate that host animals may utilize nutrients more effectively during the combination treatment of TPN with DFMO, showing increased albumin levels as compared to the rats treated with TPN alone. Correlation of hepatic ODC to plasma albumin is not apparent in this study. Based on the result from the combined treatment of TPN with DFMO, the absence of significant DFMO inhibition of hepatic ODC at 1000 mg/kg/day would suggest that hepatic polyamine production is maintained and synthetic processes are not affected. However, in RI rats, TPN increased plasma albumin levels without restoring hepatic ODC levels. The rate of albumin degradation may play an important role in regulating plasma albumin levels.[13]

The rationale for administering DFMO as a constant infusion is based on the extremely short half-life and rapid turnover rate of ODC. To effectively inhibit this enzyme, constant availability of DFMO may be necessary to inhibit tumor polyamine biosynthesis. Our result suggests that the 400 to 500 mg/kg/day dose of this agent given as a continuous infusion could produce significant antitumor activity without suppressing platelet counts, which is the major toxicity symptom of DFMO.[14] This approach may lead to an adjuvant use of this agent to other agents as well as use in maintenance therapy. Based on the above results derived from the animal studies, we are currently initiating a clinical study employing TPN combined with a constant infusion of DFMO.

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Table 4
EFFECT OF SIMULTANEOUS ADMINISTRATION OF TPN AND DFMO

Treatments	No.	Tumor weight (g)	Plasma albumin[b]	Liver ODC[c]	Tumor ODC[c]	Tumor putrescine[d]	RBC putrescine[e]
Control[a]	5	30.5 ± 4.4	3.87 ± 0.37	10.9 ± 5.6	6.1 ± 2.1	40 ± 18	1.1 ± 0.1
TPN	5	34.7 ± 11.7	4.18 ± 0.48	7.8 ± 2.3	4.7 ± 1.6	77 ± 10	2.2 ± 0.5
TPN + DFMO	4	29.1 ± 4.6	4.90 ± 0.41[f]	9.1 ± 2.4	1.4 ± 0.4[f]	4.5 ± 4.2[f]	0.5 ± 0.2[f]

[a] Tumor-bearing control rats were fed chow ad libitum.
[b] g/100 mℓ.
[c] nmol CO_2/hr/g wet tissue.
[d] nmol/g wet tissue.
[e] nmol/mℓ packed RBC.
[f] Means differ significantly as compared with TPN-treated rats.

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Index

INDEX

A

AAA, see Amino acid analyzer
AAF, see 2-Acetylaminofluorene
ABA, see Abscisic acid
Abscisic acid (ABA), 102, 108, 125, 133—134, 136, 138
ACC, see 1-Aminocyclopropane-1-carboxylic acid
Acetobacteria sp., 5
2-Acetylaminofluorene (AAF), 194
N-Acetylated spermine, 24
Acetylpolyamines, 244—226, see also specific types
Acetylputrescine, 226, 241
Acetylputrescine deacetylase, 222
Acetylspermidine, 225, 241
Acetylspermine, 236
N′-Acetyl transferase, 7
Acromegaly, 165
Actinomycin D, 129
Acute renal failure (ARF), 167, 172
Acyclovir, 20, 22
ADC, see Arginine decarboxylase
Adenosine monophosphate (AMP), 123
S-Adenosyl-1,8-diamino-3-thiooctane, 23
S-Adenosylmethionine (SAM), 5, 6, 124
  plant hormones and, 130—133
  stress and, 100
S-Adenosylmethionine decarboxylase (SAMDC), 5, 7, 19, 133, 134
  bacteria and, 16
  as chemotherapy, 262—274
  in *Escherichia coli*, 64
  inhibitors of, 17, 262—263, see also specific types
  in *Saccharomyces cerevisiae*, 68
  viruses and, 17
Adenosylmethionine synthetase, 65
Adenoviruses, 8, 18, see also specific types
Adenylate cyclase, 91
Adriamycin, 239
Adult respiratory distress syndrome (ARDS), 167, 171—172
African sleeping sickness, 20, 175
African violet, 115
Aging, 227, 237, 238
Agmatinase (AUH), 86, 91, 93
Agmatine, 5, 100
Agmatine iminohydrolase, 5
Agmatine ureohydrolase, 64
Agrobacterium tumefaciens, 5, 112
AHH, 189
AIDS, 20—22, 175
Alkaloids, 139, 154, see also specific types
Amber suppressor, 16
Amidinotransferase, 86
Amine oxidase, 222
Amino acid analyzer (AAA), 220—222
Amino acids, 100, 165—166, see also specific types
α-Aminobutyric acid, 100, 151
1-Aminocyclopropane-1-carboxylic acid (ACC), 124, 131, 132
Aminoguanidine, 18, 239
3-Aminopropionaldehyde, 7
Aminopropyl derivatives, 13, see also specific types
Aminopropyltransferases, 6, 37, 42, 49, see also specific types
AMP, see Adenosine monophosphate
Amylase, 128—130
Animal cells, 6—7, see also specific types
Animal hormones, 136—138, see also specific types
Antibacterial agents, 20, 23—24, see also specific types
Antibodies, 14, see also specific types
Antigens, 184, 227, 243, see also specific types
Antimicrobial agents, 20, see also specific types
Antitumor effect, 21, see also specific types
Antiviral activity, 20—22, see also specific types
Antizymes, 6, 76, 77, 94, 128, see also specific types
Archaebacteria, 53, 58, see also specific types
ARDS, see Adult respiratory distress syndrome
ARF, see Acute renal failure
Arginase, 5
Arginine, 5, 136
  in *Escherichia coli*, 87—90
  growth disorders and, 165
  metabolism of, 7
  plant hormones and, 126, 127, 129
  stress and, 100
  viruses and, 17
Arginine decarboxylase (ADC), 5, 77, 86, 93, 136
  bacteria and, 16
  cloning and, 138
  cultures and, 134
  in *Escherichia coli*, 64, 74
  inhibition of, 102
  metabolism and, 105
  plant differentiation and, 113, 114
  plant growth and, 108—112
  plant hormones and, 126, 127, 129, 130, 132—134
  plant tumors and, 112
  stress and, 100, 103, 104
  synthesis of, 87
Arginine/ornithine catabolic pathway, 90—94
Arginine succinyltransferase, 86
Arginine ureohydrolase, 16
Aspergillus nidulans, 69
Assays, 244, see also specific types
  in clinical medicine, 220—224
  coupled enzymatic, 52
  differential, 223
  enzymatic, 222—223
  for propylamine transferase reactions, 51—52
  total, 222—223
Atmospheric pollutants, 104, see also specific types
AUH, see Agmatinase
Autocrine, 205
Autointoxication, 167
Auxins, 122—123, 125—127, 134, 135, see also specific types

Auxotrophs, 16

B

Bacillus acidocaldarius, 38
Bacillus caldolyticus, 38
Bacillus stearothermophilus, 36, 38
Bacteria, 4, 16—17, see also specific types
Bacteriophages, 16, see also specific types
Beckwith-Wiedemann syndrome (BWS), 165
Benzyladenine, 127
Benzylaminopurine, 123, 136
Betacyanine, 128
Betaines, 100, see also specific types
Biochemical functions of polyamines, 14—20, 42—44, see also specific types
Biodegradative enzymes, 6, see also specific types
Biological fluids, 236—238, see also specific types
Biopsy, 244
Bis(guanylhydrazones), 262—268, see also specific types
  antimitochondrial effects of, 265—266
  antiproliferative effects of, 268—270
  cellular accumulation of, 264—265
  clinical studies with, 273—274
  methylglyoxal, see Methylglyoxal bis(guanylhydrazone)
  ODC and, 267—268
  polyamine acetylase and, 267—268
  resistance to, 269—270
Bleomycin, 22
Bone marrow, 226
Brain tumors, 224—225, 236, 237, 241
Breast cancer, 225, 241, 243
Breast tumors, 239
Buoyant density, 14
BWS, see Beckwith-Wiedemann syndrome

C

Cadaverine, 13, 128, 131, 137
Calcium, 103
Caldariella acidophila, see *Sulfolobus solfataricus*
Caldopentamine, 5, 37
Canaline, 126, 127
Canavanine, 126, 127, 138
Canavelmine, 37
Cancer, 11, 21, 22, 175, 238—241, see also specific types
  biology of, 184
  breast, 225, 241, 243
  cervical, 243
  characteristic changes in polyamines in, 224—225
  chemoprevention of, 261—262
  colon, 243
  colorectal, 241, 243
  DFMO in treatment of, 287—291
  diagnosis of, 244
  early detection of, 236, 243—244
  erythrocytes and, 240—241, 243
  esophageal, 243
  interferons in treatment of, 287—291
  liver, 243
  lung, 225, 241, 243
  ovarian, 225, 241
  prostaglandins and, 184
  prostatic, 225, 243
  solid, 241
  staging of, 243
  stomach, 225, 243
  treatment for, 241—243, see also Chemotherapy; specific types
  urine in, 238—240, 244
  uterine, 225, 243
  X factors of, 167
Carbonylcyanide-*m*-chloro-phenilhydrazone, 115
Carcinoembryonic antigen (CEA), 227, 243
Carcinogenesis, see also Cancer; specific types
  ionizing radiation and, 203
  in mouse skin, 186—195
  polyamine metabolism in, 203
  in rodent liver, 193—197
  two-stage, 186—195
  UV irradiation and, 203
Carcinoma, 243, see also Cancer; specific types
Carnitine, 265—266
Carrots, 114, 116, 153
CAT, see Catalase
Catabolism, 7
Catabolite repression, 86, 90, 91
Catalase (CAT), 190
CEA, see Carcinoembryonic antigen
Cell cultures, 134—135
Cell cycle, 109—112, 126
Cell differentiation, 134
Cell division, 111—112, 126, 130, 133, 138
Cell growth, 184
Cellular DNA, 18
Cellular transformation, 11, see also specific types
Centrifugation, 14, see also specific types
Cereals, 102
Cervical cancer, 243
Chemiluminescence, 223
Chemotherapy, 4, 22, 225, 241, 253—276, see also specific types
  ODC in, 262—274
  inhibitors of, 253—262
Chicory roots, 113
Chlorophyll, 131, 136
Chloroplasts, 138
Chromatin, 129
Chromatography, 223, 224, see also specific types
Chromosomal damage, 18, 184, see also specific types
Chromosomal diseases, 165—166, see also specific types
Cinnamoyl-putrescines, 139
Cirrhosis of liver, 227
Citrulline decarboxylase, 112
Clinical medicine assays, 220—224
Cloning, 138
Clostridium thermohydrosulfuricum, 36, 38, 44, 48

CMV, see Cytomegalovirus
Colon cancer, 243
Colon tumors, 241
Colorectal cancer, 225, 241, 243
Coma, 174
Conformational changes, 17
Conjugation, 90
Corn, 116, 153
Coupled enzymatic assays, 52, see also specific types
Creatinine, 24
Cryptosporidia infections, 20
Cyclic AMP, 77, 79, 80, 86, 90—91
  drug addiction and, 175
  enzyme synthesis and, 92
Cycloheximide, 102
Cysteine, 189
Cystic fibrosis, 227
Cystinuria, 166
Cytoheximide, 128
Cytokinins, 108, 123—124, 127—128, 134, 136, 138
Cytomegalovirus (CMV), 4, 6, 8, 18, 19, 22, 24
Cytostatic drugs, 258—260, see also specific types
Cytotoxic drugs, 22, see also specific types

D

2,4-D, see 2,4-Dichlorophenoxy-acetic acid
DAO, see Diamine oxidase
Datura innoxia, 113
DCHA, see Dicyclohexylamine
Deafness, 24, 174—175
Decarboxylases, 5, see also specific types
DENA, see Diethylnitrosamine
Desulfurococcus mobilis, 36
DFMA, see α-Difluoromethylarginine
DFMO, see α-Difluoromethylornithine
Diamine oxidase (DAO), 128, 168, 174, 223, 239, 263—264
Diamines, 243, see also specific types
1,4-Diaminobutane, 51
1,3-Diaminopropane, 5, 37, 51, 53—55, 57, 131
2,4-Dichlorophenoxy-acetic acid (2,4-D), 122, 126, 134
Dicyclohexylamine (DCHA), 58, 113, 114
Dicyclohexylammonium sulfate, 23
Diethylnitrosamine (DENA), 194, 195
Differential assay, 223
α-Difluoromethylarginine (DFMA), 23, 102
  cultures and, 134
  plant differentiation and, 114
  plant growth and, 112
  plant hormones and, 126, 129, 130, 132
α-Difluoromethylornithine (DFMO), 18—20, 43, 139
  as chemotherapy, 253, 260, 261
  clinical efficacy of, 21
  clinical studies of, 287—291
  cultures and, 134, 135
  genetic diseases and, 165
  interferon combined with, 287—291
  metabolism and, 105
  plant differentiation and, 113, 114
  plant growth and, 111, 112
  plant hormones and, 126, 129, 130
  polyamine metabolism changes and, 293—299
  resistance to, 150, 151
  sleeping sickness and, 175
  stress and, 102
  TPN and, 298
  transplants and, 147
  viruses and, 13
7,12-Dimethylbenz(a)anthracene (DMBA), 186, 187, 189
2,4-Dinitrophenol (DNP), 115
Disease, see specific types
DMBA, see 7,12-Dimethylbenz(a)anthracene
DMV, 21
DNA, 14, 16, 138
  cellular, 18
  chloroplast, 138
  synthesis of, 7, 8, 188, 189, 198
DNA-binding proteins, 20, see also specific types
DNA polymerase, 17, 20
DNase, 18, 138
DNA viruses, 7—10, see also specific types
DNP, see 2,4-Dinitrophenol
Donors, 90
Dormancy of plants, 108, 109, 111
Drug addiction, 175
Duchenne muscular dystrophy, 166
Dysimmunity, 173—174

E

Eimeria tenella, 20
Electron capture detectors, 223
Embryogenesis, 135
Encephalopathies, 167, see also specific types
Endogenous concentrations, 137
Endonuclease, 18
End-product inhibition, 86
Energy metabolism, 184
Enzymatic assays, 52, 222—223, see also specific types
Enzymes, see also specific types
  biodegradative, 6, see also specific types
  of *Escherichia coli*, 65, see also specific types
  in mutants, 89, 93
  stabilization of, 236
  synthesis of, 90—94
  thermophilic, 58, see also specific types
  virus-coded, 17
EPP, see Ethyl phenylpropiolate
Erythrocytes, 226, 294
  cancer and, 240—241, 243
  spermine in, 166
Escherichia coli, 5, 23, 56, 58
  enzymes of, 65, see also specific types
  feedback inhibition and, 74—76
  nitrogen source in, 87—90
  nucleotides and, 76
  polyamine-deficient, 65—66

  polyamine synthesis deficiency in, 64—66
  potassium and, 76
  putrescine biosynthetic genes in, 73—82
  repression effects and, 74—76
Esophageal cancer, 243
Ethylene, 124—125, 127—133, 136, 138
Ethyl phenylpropiolate (EPP), 187, 188
Eukaryotes, 5, 184
Excess in polyamines, see Hyperpolyaminemia
Exonuclease, 18
Experimental oncology, 186—197

F

Fanconi's syndrome, 166
Feedback inhibition, 74—76
Ferritin, 227
Fetoprotein, 227, 243
Fibroblasts, 8, 9, 236
Fibrosarcoma, 294
5-Fluorodeoxyuridine (FUdR), 9
Fragmentography, 223
FUdR, see 5-Fluorodeoxyuridine
Fungal parasites, 105

G

GABA, see γ-aminobutyric acid
γ-aminobutyric acid (GABA), 86, 91, 100, 151, 154
Gas chromatography (GC), 223—224
GC, see Gas chromatography
Genes, 79, 138, 256, see also specific types
Genetic diseases, 165—166, see also specific types
Genetic loci, 16
Genital tract tumors, 239
Genome analysis, 8
Giardia lambia, 20
Gibberellic acid, 152
Gibberellins, 108, 124, 128—130, 136, 138, see also specific types
Glucose, 93
Glutamic acid, 189
Glutamine, 126
Glutamine synthase, 86
Glutathione, 189
Glycine, 189
Growth disorders, 164—165, see also specific types
Growth hormone (GH), 164—166
Growth regulators, 125, see also specific types
GTP, see Guanosine triphosphate
Guanosine tetraphosphate, 76
Guanosine triphosphate (GTP), 76

H

Haemophilus influenzae, 4
Haemophilus parainfluenzae, 69, 164
Halophilic archaebacteria, 39, 49
Heat shock protein, 104
HeLa cells, 17
Helianthus sp., 111—113
Helianthus tuberosus, 108—111, 126, 127
Hematological malignancies, 225
Hepatomas, 197
Herpes simplex virus (HSV), 6, 7, 17, 18, 22
Herpesviruses, 4, see also specific types
hGH, see Human growth hormone
High-performance liquid chromatography (HPLC), 24, 52, 220—222, 224
Homocaldohexamine, 38
Homocaldopentamine, 5
Homospermidine, 5, 39
Hormones, see also specific types
  animal vs. plant, 136—138
  growth, see Growth hormone
  HPA and release of, 169
  plant, see Plant hormones
HPA, see Hyperpolyaminemia
HPLC, see High-performance liquid chromatography
HSV, see Herpes simplex virus
Human growth hormone (hGH), 166
Hydrolysis, 236, 241
Hydroxycinnamic acid, 154
Hydroxycinnamic acid amides, 153
Hydroxymethylpterin, 243
Hyperpolyaminemia (HPA), 166—174
Hyperproliferative skin, 272—273

I

IAA, see Indole-3-acetic acid
IBA, see Indolebutyric acid
IBMX, see 3-Isobutyl-1-methylxantine
IFN, see Interferon
Immune system, 21
Indole-3-acetic acid (IAA), 108, 111, 122, 126, 127, 136
Indolebutyric acid (IBA), 113, 126
Influenza A, 10
Inhibition studies, 55—58, see also specific types
Interferon, 22, 287—291, see also specific types
Interrupted-mating, 90
Intrauterine growth retardation (IUGR), 165, 175
5-Iodouridinedeoxyribose (IUDR), 21
Ionizing radiation, 203
3-Isobutyl-1-methylxantine (IBMX), 188
Isoputreamine, 241
Isopycnic centrifugation, 14
IUDR, see 5-Iododouridinedeoxyribose
IUGR, see Intrauterine growth retardation

K

Kdalton matrix protein, 20
Kinetics, 55—58, see also specific types
Kinetin, 123, 127, 136
Kinky hair disease, 227
Klebsiella pneumoniae, 23

L

Lactobacilli, 164, see also specific types
Lactobacillus casei, 4
LDC, see Lysine decarboxylase

Lectins, 236, see also specific types
Leishmania sp., 20
Leukemia, 13, 21, 200, 225, 239, 243, see also specific types
Liver cancer, 243
Liver carcinogenesis, 193—197
Liver cirrhosis, 227
Liver disorders, 171
Liver failure, 174
L1210 mouse leukemia cells, 13
Lowe's syndrome, 166
Lung cancer, 225, 241, 243
Lung cells, 14
Lupus, 227
Lymphocytes, 236
Lymphomas, 200, 225, 241, see also specific types
Lysate, 88
Lysine, 136, 137
Lysine decarboxylase (LDC), 13, 65

M

Macrophages, 13
Magnesium, 7, 103
Malignancies, 24, 225, 236, see also specific types
Malnutrition, 294, 296
Mammalian neoplastic cells, 197—201
Markers, 4, 24—25, 243, see also specific types
Mass fragmentography, 223
Mass spectrometry (MS), 39, 223
Maternal-infant interplay, 164
Maternal nutrition, 164
MC, see 3-Methylcholanthrene
MDAB, see 3′-Methyl-4-dimethylaminoazobenzene
Medullablastoma, 237
Melanoma, 243
Membranes, 131, 133, 136, 138, 184, see also specific types
Menkes kinky hair disease, 227
Menstrual cycle, 227, 237, 238
Messenger RNA, 79
Metabolic diversion, 139
Metabolism, 129, 138
  arginine, 7
  energy, 184
  nucleic acid, 131
  rapid, 137
  stress and, 100—104
Metabolism of polyamines
  abnormalities of, 166, 204
  in brain, 164
  in carcinogenesis, 203
  changes in tumor-bearing hosts, 293—299
  clinical perturbations in, 167
  in cultured mammalian neoplastic cells, 197—201
  disturbances in, 165
  in experimental oncology, 186—197
  fungal parasites and, 105
  liver disorders and, 171
  in neoplastic cells, 204
  programmed deregulation of, 204—205
Methanobacterium thermoautotrophicum, 36
Methionine, 5, 131
3-Methylcholanthrene (MC), 194
3′-Methyl-4-dimethylaminoazobenzene (MDAB), 196
Methylglyoxal bis(butylamidinohydrazone), 23
Methylglyoxal bis(guanylhydrazone) (MGBG), 17, 18, 21, 23, 110
  cell cycle and, 112
  as chemotherapy, 262, 263
  cultures and, 134
  plant differentiation and, 113, 114
  plant hormones and, 126, 127, 132, 133
  resistance to, 149—151
  transplants and, 174
α-Methylornithine, 113
5′-Methylthioadenosine (MTA), 5, 6, 52, 53, 56, 57, 124, 202
Methylthioriboside (MTR), 124
Metronidazole, 25
Mezerein (MZ), 186—189, 192
MGBG, see Methylglycoxal bis(guanylhydrazone)
Microbial infections, 24—25, see also specific types
Microbial mutants, 63—69, see also specific types
Microbiology, 20—25
Mink lung cells, 14
Mitochondrial functions, 17
Mitotic activity, 135
Monoclonal antibodies, 14, see also specific types
α-Monofluoromethylornithine, 23
α-Monofluoromethylputrescine, 23
Morphine, 175
Morphogenesis, 135
Mouse leukemia, 200
Mouse lymphoma, 200
Mouse skin carcinogenesis, 186—195
MS, see Mass spectrometry
MTA, see 5′-Methylthioadenosine
MTR, see Methylthioriboside
Multiple myeloma, 225
Murine CMV, 8
Murine sarcoma, 12
Muscular dystrophy, 166
Mutants, 91, 138—139, 147—158, see also specific types
  analysis of, 148, 152—154
  direct selection of for polyamine alterations, 149—152
  drug-resistant, 21
  enzymes in, 89, 93
  microbial, 63—69
  polyamine synthesis regulation and, 154—157
  rps, 16
  selection of for nonpolyamine phenotypes, 152—154
Mycoplasma arginini, 12
Mycoplasma dispar, 13
Mycoplasma hyorhinis, 12
Mycoplasma laidlawii, 13
Mycoplasma orale, 12
Mycoplasma sp., 8, 12—14

Mycoplasma suispneumoniae, 13
Myocardiopathy, 171
Myotonic muscular dystrophy, 166
Myxoviruses, 6, see also specific types
MZ, see Mezerein

N

Neisseria perflava, 4
Neoplastic cells, see also specific types
  cultured mammalian, 197—201
  ODC in, 200—201
  polyamine metabolism in, 204
  regulation of proliferation of, 205
Nerve tumor cells, 198—200
Neuropsychiatric disorders, 175, see also specific types
Neurospora crassa, 69
Newcastle disease virus, 10
Nicotiana glauca, 112
Nicotiana langsdorffii, 112
Nicotiana sp., 154
Nicotiana sylvestris, 153
Nicotiana tabacum, 149, 152
Nicotine, 139, 154
Nitrogen
  availability of, 86
  degradation of, 91
  detectors of, 223
  in *Escherichia coli*, 87—90
NMR, see Nuclear magnetic resonance
Nonspecific vaginitis (NSV), 25
Nontumor diseases, 227, see also specific types
Norspermidine, 5, 37, 39
Norspermine, 5
NSV, see Nonspecific vaginitis
Nuclear magnetic resonance (NMR), 38—41
Nucleic acids, 131, 132, 138, see also specific types
Nucleocapsid, 17
Nucleoside phosphorylase, 14
Nucleotides, 10, 76, 184, see also specific types
Nutrition, 296
  cancer and, 244
  maternal, 164
  stress and, 103—104
  total parenteral, 293—299

O

ODC, see Ornithine decarboxylase
Oncology, see also Cancer; specific types
  human, 201—202
  polyamine metabolism in, 186—197
  two-stage carcinogenesis and, 186—195
Oncornaviruses, 12, see also specific types
Operon, 93
Ornithine, 5, 136
  in *Escherichia coli*, 87—90
  plant hormones and, 127, 129
  stress and, 100
Ornithine aminotransferase, 87
Ornithine decarboxylase (ODC), 5, 77, 93, 136
  antizymes of, 6, 76
  in *Aspergillus nidulans*, 69
  bis(guanylhydrazone) effects on, 267—268
  cancer and, 236, 244
  carcinogenesis and, 188—193, 195
  in chemotherapy, 253—262
  cloning and, 138
  in cultured mammalian neoplastic cells, 200—201
  drug addiction and, 175
  in *Escherichia coli*, 64, 74, 87
  hepatic, 194
  hepatoma and, 197, 198
  in human oncology, 202
  inhibitors of, 19, 253—255, see also specific types
    clinical studies of, 260—261
    combination of other agents with, 270—272
    cytostatic drugs combined with, 258—260
    resistance to, 253—255
  leukemia and, 200
  lymphoma and, 200
  maternal-infant interaction and, 164
  metabolism and, 105
  nerve tumor cells and, 198—200
  periodontal disease and, 174
  plant differentiation and, 113, 114
  plant growth and, 108—112
  plant hormones and, 126, 128, 129
  plant tumors and, 112
  radiation and, 203
  in *Saccharomyces cerevisiae*, 68
  stress and, 100, 104
  synthesis of, 87
  synthetic, 6
  TPA and, 189, 192, 193
  tumors and, 296
  viruses and, 17
Ornithine transcarbamylase deficiency (OTCD), 166
Osmotic regulatory mechanisms, 76
Osmotic stress, 100—102
OTCD, see Ornithine transcarbamylase deficiency
Ovarian cancer, 225, 241

P

Paclobutrazol, 130
Parasites, 21, 105, see also specific types
Parenteral nutrition, 293—299
Pasteurella tularensis, 4
PAT, see Putrescine aminotransferase
Pentamidine, 175
Periodontal disease, 174
Periplasmic space, 77
Petunia sp., 154
PGTX, see Toxemia
pH, 103, 116, 157
PHA, see Phytohemagglutinin
Pheochromocytoma, 171
Phloem, 138
Phorbol esters, 187, see also specific types
Phosphorus, 103

Phosphorylation, 20
Phylogenesis, 53
Physiochemical stress, 100—104, see also specific types
Physiological function of polyamines, 14—20, see also specific types
Phytochromes, 130
Phytohemagglutinin (PHA), 191
Picornaviruses, 6, 10, see also specific types
Ping-pong mechanisms, 56
Pinus radiata, 111, 113
Pisum sativum, 152
Plant differentiation, 112—114, 122
Plant dormancy, 108, 109, 111
Plant growth, 108—112, 114—117, 122
Plant hormones, 122—125, see also specific types
  animal hormones vs., 136—137
  direct interactions of polyamines and, 125—134
  functional characteristics of, 136—139
  indirect putative interactions with, 134—136
Plant tumors, 112, see also specific types
Plasmids, 77—79, see also specific types
Plasmodium berghei, 22
Plasmodium sp., 20
Pneumocystis carinii, 14, 20, 22, 175
Pneumonia, 20, 22, 175, see also specific types
Pollutants, 104, see also specific types
Polyamine acceptors, 53, see also specific types
Polyamine acetylase, 267—268
Polyamine antimetabolite, 272—273
Polyamine inhibitors, 20, see also specific types
Polyamine oxidases, 7, 128, 223, see also specific types
Polyamine precursors, 139, see also specific types
Polyamine synthase, 19
Polycythemia vera, 227
Potassium, 76, 103, 104
Pregnancy, 227
  Sheehan's fatty liver of, 174
  toxemia of, 167, 169, 172—173
Prematurity, 175
Prokaryotes, 5
Prolactin, 169
Proline, 100
Propylamine acceptors, 54, see also specific types
Propylamine transferase reactions, 47—60, see also specific types
  assays for, 51—52
  kinetics of, 55—58
  mechanisms of, 55—58
  Sulfolobus solfataricus and, 52—55
Prostaglandins, 184, see also specific types
Prostatic cancer, 225, 243
Prostatic tumors, 239
Proteins, 138, see also specific types
  activators of, 76—77
  basic, 94
  DNA-binding, 20
  heat shock, 104
  inhibitors of, 76—77
  kdalton matrix, 20
  late, 19
  S1, 16
  synthesis of, 8, 42, 132
Protoplasts, 132, 135, 136
PrrD, see Pyrroline dehydrogenase
Pseudomonas aeruginosa, 23
Psoriasis, 165, 227, 261
PTC, 164
Pulmonary infections, 175
Purification, 52—55, see also specific methods
Putrescine, 53, 54, 86, 137
  accumulation of, 102
  in *Aspergillus nidulans*, 69
  bacteria and, 4, 16
  in brain tumors, 224—225
  cancer and, 236
  carcinogenesis and, 188
  depletion of, 255—258
  in *Escherichia coli*, 64
  in *Haemophilus parainfluenzae*, 69
  in hematological malignancies, 225
  inhibition of, 154
  malnutrition and, 296
  mutants and, 138
  in *Neurospora crassa*, 69
  plant differentiation and, 114
  plant growth and, 108, 115
  plant hormones and, 126, 127, 129, 131, 133
  resistance to, 151
  in *Saccharomyces cerevisiae*, 66
  separation of, 52
  stress and, 100, 102—104
  Sulfolobus solfataricus and, 51
  synthesis of, 114
  tumors and, 296
  utilization of, 151
Putrescine aminotransferase (PAT), 65, 86, 87, 89
Putrescine biosynthetic genes, 73—82, 77—80
Putrescine transcarbamylase, 5
Pyrodictium brockii, 36
Pyrrolidine alkaloids, 154, see also specific types
Pyrroline-5-carboxylate dehydrogenase, 87
Pyrroline dehydrogenase (PrrD), 86, 87, 90, 91, 93

R

Radiation, 203, 241, 259
Radioimmunoassay (RIA), 224, 243
Red blood cells, see Erythrocytes
Repression effects, 74—76
Research problems, 116—117
Reverse-phase HPLC, 24
Reye's syndrome, 169, 171, 174
RIA, see Radioimmunoassay
Ribonuclease, 227
Ribosomal subunits, 16, see also specific types
RNA, 138
  messenger, 79
  synthesis of, 7, 8, 91, 126
RNA bacteriophage, 16
RNA polymerase, 17

RNase, 136, 138
RNA viruses, 10—12, see also specific types
Rodent liver carcinogenesis, 193—197
Ross sarcoma, 12
Rps mutation, 16

S

Saccharomyces cerevisiae, 48, 66—69
Salinity, 104, 136
SAM, see *S*-Adenosylmethionine
SAMDC, see *S*-Adenosylmethionine decarboxylase
Sarcoma, 12, 294, see also specific types
SAT, see Spermidine acetyltransferase
Scorzonera hipanica, 112, 126
Scorzonera sp., 112
Semliki Forest virus (SFV), 10
Senescence, 128, 130—134, 136
Sensorineural deafness, 24
Sephritis, 227
Sex differences in polyamine levels, 237—238
SFV, see Semliki Forest virus
Sheehan's fatty liver of pregnancy, 174
Shock, 171
Shock heart, 167
Sickle cell disease (SSD), 166, 227
Sleeping sickness, 20, 175
SOD, see Superoxide dismutase
Solid cancer, 241
Solid tumors, 225
Sorbitol, 102
SP, see Spermine
SPD, see Spermidine
Spectrometry, mass (MS), 39, 223, see also specific types
Spermidine (SPD), 50, 53, 54, 57, 165, 170, 172, 174
  analogues of, 21
  in *Aspergillus nidulans*, 69
  bacteria and, 4, 16
  cancer and, 236
  carcinogenesis and, 188
  depletion of, 255—258
  in *Escherichia coli*, 64
  in hematological malignancies, 225
  plant growth and, 108, 115
  plant hormones and, 126
  in *Saccharomyces cerevisiae*, 66
  separation of, 52
  spermine ratio to, 8
  stress and, 100
  Sulfolobus solfataricus and, 51
  synthesis of, 51
  tissue levels of, 164
Spermidine acetyltransferase (SAT), 236
Spermidine synthase, 5, 53, 55, 57, 58
  from bovine brain, 56
  in *Escherichia coli*, 64
  inhibitors of, 274—276, see also specific types
  in *Saccharomyces cerevisiae*, 68
Spermine (SPM), 50, 51, 54, 165, 166, 169, 170, 172, 174
  N-acetylated, 24
  in *Aspergillus nidulans*, 69
  bacteria and, 5
  cancer and, 236
  carcinogenesis and, 188
  erythrocyte, 166
  plant growth and, 108, 115
  plant hormones and, 126
  in *Saccharomyces cerevisiae*, 66
  separation of, 52
  spermidine ratio to, 8
  stress and, 100
  tissue levels of, 164
Spermine synthase, 6, 55, 57, 68—69, 274—276
SPM, see Spermine
S1 protein, 16
SSD, see Sickle cell disease
Stomach cancer, 225, 243
Stomach tumors, 239, 241
Streptomycin, 16
Stress, 122, 134, 136, see also specific types
  cancer and, 244
  metabolism and, 100—104
  nutritional, 103—104
  osmotic, 100—102
  pH, 103, 157
  physiochemical, 100—104
  response to, 99—105
  temperature, 104
  water, 100—102
Stress metabolism, 100
Subcellular distribution of polyamines, 114—116
Substrates, 86, 93, see also specific types
Sudden cardiac death, 171
Sulfolobus acidocaldarius, 39, 42
Sulfolobus solfataricus, 44, 48—55
Superoxide dismutase (SOD), 199
Symmetrical polyamines, 48—51, see also specific types
Symptomatic infections, 24, see also specific types
Synthesis of polyamines, 86
  in animal cells, 6—7
  microbial mutants deficient in, 63—69
  in microbiology, 20—25
  regulation of, 154—157, 164
Systemic lupus erythematosis, 227

T

Temperature stress, 104
12-*O*-Tetradecanoylphorbol 13-acetate (TPA), 186—193, 197, 199
Thalidomide, 165
Therapeutic index, 20
Thermine, 39, 47
Thermophiles, 35—44, see also specific types
Thermophilic archaebacteria, 39, 48—51, see also specific types
Thermophilic enzymes, 58, see also specific types
Thermophilic eubacteria, 36—39, see also specific types

Thermoplasma acidophilum, 39
Thermoproteus tenax, 36
Thermospermine, 5
Thermus aquaticus, 36
Thermus flavus, 36
Thermus sp., 36
Thermus thermophilus, 5, 36, 37, 39, 44, 50
Thin-layer chromatography (TLC), 224
Thymidine kinase, 9
Tissue cultures, 134—135
TLC, see Thin-layer chromatography
Tomato, 116
Tosylphenyladanyl chloromethylketone (TPCK), 186, 187
Total assay, 222—223
Total parenteral nutrition (TPN), 293—299
Toxemia, 167, 169, 172—173
Toxic encephalopathies, 167
Toxicity, 167—170, see also specific types
TPA, see 12-*O*-Tetradecanoylphorbol 13-acetate
TPCK, see Tosylphenylalanyl chloromethylketone
TPN, see Total parenteral nutrition
Transduction, 88—89
Translocation of polyamines, 114—116, 137—138
Transplantations, 174
Trauma, 174
Trichomonus vaginitis, 25
Trypanosomes, 14
Trypanosomiasis, 22
Tumor markers, 224—229, 236, see also specific types
Tumor promoters, 187, see also specific types
Tumors, 239, 241, 269, see also specific types
  brain, 224—225, 236, 237, 241
  malnutrition and, 296
  nerve, 198—200
  ODC and, 296
  plant, 112
  polyamine links with, 185
  polyamine metabolism changes and, 293—299
  regression of, 241
  solid, 225
Tumor viruses, 239, see also specific types
Turner's syndrome, 165
Two-stage carcinogenesis, 186—195, see also specific types

U

Unusual polyamines, 39—42, see also specific types
Uptake of polyamines, 114—116, 137—138
Uremia, 168, 170, 174
Urine in cancer, 238—240, 244
Uterine cancer, 225, 243
UV irradiation, 203

V

Vaccinia virus, 6
Vaginitis, 25
Varicella-zoster virus, 14
Veillonella alcalescens, 4
Vigna sp., 154
Viruses, 4, 6—12, 132, see also specific types
  DNA, 7—10, see also specific types
  DNA polymerase and, 20
  enzymes coded with, 17
  fibroblasts and, 236
  functions of polyamines and, 17—20
  RNA, 10—12, see also specific types
  RNA polymerase and, 17
  tumor, 239
Vomiting, 175

W

Water stress, 100—102

X

X factors of cancer, 167
Xylem, 138

Z

Zeatin, 115, 123

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